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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/47, C12N 15/12

A1

(11) International Publication Number: WO 98/14475

(43) International Publication Date: 9 April 1998 (09.04.98)

(21) International Application Number: PCT/US97/17433

(22) International Filing Date: 29 September 1997 (29.09.97)

(30) Priority Data:
08/720,484 30 September 1996 (30.09.96) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: VERTEBRATE SMOOTHENED PROTEINS

(57) Abstract

Novel vertebrate homologues of Smoothened, including human and rat Smoothened, are provided. Compositions including vertebrate Smoothened chimeras, nucleic acid encoding vertebrate Smoothened, and antibodies to vertebrate Smoothened, are also provided.

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Vertebrate Smoothened Proteins

FIELD OF THE INVENTION

The present invention relates generally to novel Smoothened proteins which interact with Hedgehog and Patched signalling molecules involved in cell proliferation and differentiation. In particular, the invention relates to newly identified and isolated vertebrate Smoothened proteins and DNA encoding the same, including rat and human Smoothened, and to various modified forms of these proteins, to vertebrate Smoothened antibodies, and to various uses thereof.

BACKGROUND OF THE INVENTION

Development of multicellular organisms depends, at least in part, on mechanisms which specify, direct or maintain positional information to pattern cells, tissues, or organs. Various secreted signalling molecules, such as members of the transforming growth factor-beta ("TGF-beta"), Wnt, fibroblast growth factor ("FGF"), and hedgehog families, have been associated with patterning activity of different cells and structures in Drosophila as well as in vertebrates [Perrimon, Cell, 80:517-520 (1995)].

Studies of Drosophila embryos have revealed that, at cellular blastoderm and later stages of development, information is maintained across cell borders by signal transduction pathways. Such pathways are believed to be initiated by extracellular signals like Wingless ("Wg") and Hedgehog ("Hh"). The extracellular signal, Hh, has been shown to control expression of TGF-beta, Wnt and FGF signalling molecules, and initiate both short-range and long-range signalling actions. A short-range action of Hh in Drosophila, for example, is found in the ventral epidermis, where Hh is associated with causing adjacent cells to maintain *wingless (wg)* expression [Perrimon, Cell, 76:781-784 (1984)]. In the vertebrate central nervous system, for example, Sonic hedgehog ("SHh"; a secreted vertebrate homologue of dHh) is expressed in notocord cells and is associated with inducing floor plate formation within the adjacent neural tube in a contact-dependent manner [Roelink et al., Cell, 76:761-775 (1994)]. Perrimon, Cell, 80:517-520 (1995) provide a general review of some of the long-range actions associated with Hh.

Studies of the Hh protein in Drosophila ("dHh") have shown that *hh* encodes a 46 kDa native protein that is cleaved into a 39 kDa form following signal sequence cleavage and subsequently cleaved into a 19 kDa amino-terminal form and a 26 kDa carboxy-terminal form [Lee et al., Science, 266:1528-1537 (1994)]. Lee et al. report that the 19 kDa and 26 kDa forms have different biochemical properties and are differentially distributed. DiNardo et al. and others have disclosed that the dHh protein triggers a signal transduction cascade that activates *wg* [DiNardo et al., Nature, 332:604-609 (1988); Hidalgo and Ingham, Development, 110:291-301 (1990); Ingham and Hidalgo, Development, 117:283-291 (1993)] and at least another segment polarity gene, *patched (ptc)* [Hidalgo and Ingham, supra; Tabata and Kornberg, Cell, 76:89-102 (1994)]. Properties and characteristics of dHh are also described in reviews by Ingham et al., Curr. Opin. Genet. Dev., 5:492-498 (1995) and Lumsden and Graham et al., Curr. Biol., 5:1347-1350 (1995). Properties and characteristics of the vertebrate homologue of dHh, Sonic hedgehog, are described by Echelard et al., Cell, 75:1417-1430 (1993); Krauss et al., Cell, 75:1431-1444 (1993); Riddle et al., Cell, 75:1401-1416 (1993); Johnson et al., Cell, 79:1165-1173 (1994); Fan et al., Cell, 81:457-465 (1995); Roberts et al., Development, 121:3163-3174 (1995); and Hynes et al., Cell, 80:95-101 (1995).

In Perrimon, Cell, 80:517-520 (1995), it was reported that the biochemical mechanisms and receptors by which signalling molecules like Wg and Hh regulate the activities, transcription, or both, of secondary signal transducers have generally not been well understood. In Drosophila, genetic evidence indicates that Frizzled ("Fz") functions to transmit and transduce polarity signals in epidermal cells during hair and bristle development. Fz rat homologues which have structural similarity with members of the G-protein-coupled receptor superfamily have been described by Chan et al., J. Biol. Chem., 267:25202-25207 (1992). Specifically, Chan et al. describe isolating two different cDNAs from a rat cell library, the first cDNA encoding a predicted 641 residue protein, Fz-1, having 46% homology with Drosophila Fz, and a second cDNA encoding a protein, Fz-2, of 570 amino acids that is 80% homologous with Fz-1. Chan et al. state that mammalian *fz* may constitute a gene family important for transduction and intercellular transmission of polarity information during tissue morphogenesis or in differentiated tissues. Recently, Bhanot et al. did describe the identification of a Drosophila gene, *frizzled2 (Dfz2)*, and predicted Dfz2 protein, which can function as a Wg receptor in cultured cells [Bhanot et al., Nature, 382:225-230 (1996)]. Bhanot et al. disclose, however, that there is no *in vivo* evidence that shows Dfz2 is required for Wg signalling.

Although some evidence suggests that cellular responses to dHh are dependent on the transmembrane protein, smoothened (dSmo), [Nusslein-Volhard et al., Wilhelm Roux's Arch. Dev. Biol., 193:267-282 (1984); Jurgens et al., Wilhelm Roux's Arch. Dev. Biol., 193:283-295 (1984); Alcedo et al., Cell, 86:221-232 (July 26, 1996); van den Heuvel and Ingham, Nature, 382:547-551 (August 8, 1996)], and are negatively regulated by the transmembrane protein, "Patched" [(Hooper and Scott, Cell, 59:751-765 (1989); Nakano et al., Nature, 341:508-513 (1989); Hidalgo and Ingham, supra; Ingham et al., Nature, 353:184-187 (1991)], the receptors for Hh proteins have not previously been biochemically characterized. Various gene products, including the Patched protein, the transcription factor cubitus interruptus, the serine/threonine kinase "fused", and the gene products of *Costal-2, smoothened (smo)* and *Suppressor of fused (Su(fu))*, have been implicated as putative components of the Hh signalling pathway.

Prior studies in Drosophila led to the hypothesis that *ptc* encoded the Hh receptor [Ingham et al., Nature, 353:184-187 (1991)]. The activity of the *ptc* product, which is a multiple membrane spanning cell surface protein referred to as Patched [Hooper and Scott, supra], represses the *wg* and *ptc* genes and is antagonized by the Hh signal. Patched was proposed by Ingham et al. to be a constitutively active receptor which is inactivated by binding of Hh, thereby permitting transcription of Hh-responsive genes. As reported by Bejsovec and Wieschaus, Development, 119:501-517 (1993), however, Hh has effects in *ptc* null Drosophila embryos and thus cannot be the only Hh receptor. Accordingly, the role of Patched in Hh signalling has not been fully understood.

Goodrich et al. have isolated a murine *patched* gene [Goodrich et al., Genes Dev., 10:301-312 (1996)]. Human *patched* homologues have also been described in recently published literature. For instance, Hahn et al., Cell, 85:841-851 (1996) describe isolation of a human homolog of Drosophila *ptc*. The gene displays up to 67% sequence identity at the nucleotide level and 60% similarity at the amino acid level with the Drosophila gene [Hahn et al., supra]. Johnson et al. also provide a predicted amino acid sequence of a human Patched protein [Johnson et al., Science, 272:1668-1671 (1996)]. Johnson et al. disclose that the 1447 amino acid protein has 96% and 40% identity to mouse and Drosophila Patched, respectively. The human and

mouse data from these investigators suggest that *patched* is a single copy gene in mammals. According to Hahn et al., Cell, 85:841-851 (1996), analyses revealed the presence of three different 5' ends for their human *ptc* gene. Hahn et al. postulate there may be at least three different forms of the Patched protein in mammalian cells: the ancestral form represented by the murine sequence, and the two human forms. Patched is further discussed in a recent review by Marigo et al., Development, 122:1225 (1996).

Studies in Drosophila have also led to the hypothesis that Smo could be a candidate receptor for Hh [Alcedo et al., supra; van den Heuvel and Ingham, supra]. The *smoothened (smo)* gene was identified as a segment polarity gene and initially named *smooth* [Nusslein-Volhard et al., supra]. Since that name already described another locus, though, the segment polarity gene was renamed *smoothened* [Lindsley and Zimm, "The Genome of *Drosophila melanogaster*," San Diego, CA:Academic Press (1992)]. As first reported by Nusslein-Volhard et al., supra, the *smo* gene is required for the maintenance of segmentation in Drosophila embryos.

Alcedo et al., supra, have recently described the cloning of the Drosophila *smoothened* gene [see also, van den Heuvel and Ingham, supra]. Alcedo et al. report that hydropathy analysis predicts that the putative Smo protein is an integral membrane protein with seven membrane spanning alpha helices, a hydrophobic segment near the N-terminus, and a hydrophilic C-terminal tail. Thus, Smo may belong to the serpentine receptor family, whose members are all coupled to G proteins. Alcedo et al., supra, also report that *smo* is necessary for Hh signalling and that it acts downstream of *hh* and *ptc*.

As discussed in Pennisi, Science, 272:1583-1584 (1996), certain development genes are believed to play some role in cancer because they control cell growth and specialization. Recent studies suggest that *patched* is a tumor suppressor, or a gene whose loss or inactivation contributes to the excessive growth of cancer cells. Specifically, Hahn et al. and other investigators have found that *patched* is mutated in some common forms of basal cell carcinomas in humans [Hahn et al., Cell, 85:841-851 (1996); Johnson et al., supra; Gailani et al., in Letters, Nature Genetics, 13:September, 1996]. Hahn et al. report that alterations predicted to inactivate the *patched* gene product were found in six unrelated patients having basal cell nevus syndrome ("BCNS"), a familial complex of cancers and developmental abnormalities. Hahn et al. also report that the *ptc* pathway has been implicated in tumorigenesis by the cloning of the pancreatic tumor suppressor gene, *DPC4*. Vertebrate homologues of two other Drosophila segment polarity genes, the murine mammary *Wnt1* [Rijsewijk et al., Cell, 50:649 (1987)] and the human glioblastoma *GLI* [Kinzler et al., Science, 236:70 (1987)], have also been implicated in cancer.

SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel vertebrate Smoothened proteins, designated herein as "vSmo." In particular, cDNA clones encoding rat Smoothened and human Smoothened have been identified. The vSmo proteins of the invention have surprisingly been found to be co-expressed with Patched proteins and to form physical complexes with Patched. Applicants also discovered that the vSmo alone did not bind Sonic hedgehog but that vertebrate Patched homologues did bind Sonic hedgehog with relatively high affinity. It is believed that Sonic hedgehog may mediate its biological activities through a multi-subunit receptor in which vSmo is a signalling component and Patched is a ligand binding component, as well as a ligand regulated suppressor of vSmo. Accordingly, without being limited to any one theory, pathological

conditions, such as basal cell carcinoma, associated with inactivated (or mutated) Patched may be the result of constitutive activity of vSmo or vSmo signalling following from negative regulation by Patched.

In one embodiment, the invention provides isolated vertebrate Smoothened. In particular, the invention provides isolated native sequence vertebrate Smoothened, which in one embodiment, includes an amino acid sequence comprising residues 1 to 793 of Figure 1 (SEQ ID NO:2). The invention also provides isolated native sequence vertebrate Smoothened which includes an amino acid sequence comprising residues 1 to 787 of Figure 4 (SEQ ID NO:4). In other embodiments, the isolated vertebrate Smoothened comprises at least about 80% identity with native sequence vertebrate Smoothened comprising residues 1 to 787 of Figure 4 (SEQ ID NO:4).

In another embodiment, the invention provides chimeric molecules comprising vertebrate Smoothened fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises a vertebrate Smoothened fused to an epitope tag sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding vertebrate Smoothened. In one aspect, the nucleic acid molecule is RNA or DNA that encodes a vertebrate Smoothened, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under stringent conditions. In one embodiment, the nucleic acid sequence is selected from:

(a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:1) that codes for residue 1 to residue 793 (i.e., nucleotides 450-452 through 2826-2828), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 4 (SEQ ID NO:3) that codes for residue 1 to residue 787 (i.e., nucleotides 13-15 through 2371-2373), inclusive; or

(c) a sequence corresponding to the sequence of (a) or (b) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the vertebrate Smoothened. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing vertebrate Smoothened is further provided.

In another embodiment, the invention provides an antibody which specifically binds to vertebrate Smoothened. The antibody may be an agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

Another embodiment of the invention provides articles of manufacture and kits that include vertebrate Smoothened or vertebrate Smoothened antibodies.

A further embodiment of the invention provides protein complexes comprising vertebrate Smoothened protein and vertebrate Patched protein. In one embodiment the complexes further include vertebrate Hedgehog protein. The invention also provides vertebrate Patched which binds to vertebrate Smoothened. Optionally, the vertebrate Patched comprises a sequence which is a derivative of or fragment of a native sequence vertebrate Patched.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of native sequence rat Smoothened.

Figure 2 shows the primary structure of rat Smo (rSmo) and Drosophila Smo (dsmo). The signal peptide sequences are underlined, conserved amino acids are boxed, cysteines are marked with asterisks, potential glycosylation sites are marked with dashed boxes, and the seven hydrophobic transmembrane domains are shaded.

Figure 3 shows tissue distribution of SHH, Smo and Patched in embryonic and adult rat tissues. *In situ* hybridization of SHH (left column); Smo (middle column) and Patched (right column, not including insets) to rat tissues. Row E15 Sag, sagittal sections through E15 rat embryos. Rows E9, E10, E12, and E15, coronal sections through E9 neural folds, E10 neural tube and somites, E12 and E15 neural tube. Insets in Row E12 show sections through forelimb bud of E12 rat embryos. Legend- ht=heart; sk=skin; bl=bladder; ts=testes; lu=lung; to=tongue; vtc=vertebral column; nf=neural fold; nc=notocord; so=somite; fp=floor plate; vh=ventral horn; vz=ventricular zone; cm=cardiac mesoderm and vm=ventral midbrain.

Figure 4 shows the nucleotide (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) for native sequence human Smoothened.

Figure 5 shows the primary structure of human Smo (hSmo) and rat Smo (rat.Smo) and homology to Drosophila Smo (dros.smo). Conserved amino acids are boxed.

Figure 6 illustrates the results of binding and co-immunoprecipitation assays which show SHH-N binds to mPatched but not to rSmo. Staining of cells expressing the Flag tagged rSmo (a and b) or Myc tagged mPatched (c, d, and e) with (a) Flag (Smo) antibody; (c) Myc (mPatched) antibody; (b and d) IgG-SHH-N; or (e) Flag tagged SHH-N. (f) Co-immunoprecipitation of epitope tagged mPatched (Patched) or epitope tagged rSmo (Smo) with IgG-SHH-N. (g) cross-linking of ^{125}I-SHH-N (^{125}I-SHH) to cells expressing mPatched or rSmo in the absence or presence of unlabeled SHH-N. (h) Co-immunoprecipitation of ^{125}I-SHH by an epitope tagged mPatched (Patched) or an epitope tagged rSmo (Smo). (i) competition binding of ^{125}I-SHH to cells expressing mPatched or mPatched plus rSmo.

Figure 7 illustrates (a) Double immunohistochemical staining of Patched (red) and Smo (green) in transfected cells. Yellow indicates co-expression of the two proteins. (b and c) Detection of Patched-Smo Complex by immunoprecipitation. (b) immunoprecipitation with antibodies to the epitope tagged Patched and analysis on a Western blot with antibodies to epitope tagged Smo. (c) immunoprecipitation with antibodies to the epitope tagged Smo and analysis on a Western blot with antibodies to epitope tagged Patched. (d and e) co-immunoprecipitation of ^{125}I-SHH bound to cells expressing both Smo and Patched with antibodies to either Smo (d) or Patched (e) epitope tags.

Figure 8 shows a Western blot from a SDS-gel depicting the expression level of a wildtype (WT) and mutated Patched (mutant).

Figure 9 shows a model describing the putative SHH receptor and its proposed activation by SHH. As shown in the model, Patched is a ligand binding component and vSmo is a signalling component in a multi-subunit SHH receptor.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "vertebrate Smoothened", "vertebrate Smoothened protein" and "vSmo" when used herein encompass native sequence vertebrate Smoothened and vertebrate Smoothened variants (each of

which is defined herein). These terms encompass Smoothened from a variety of animals classified as vertebrates, including mammals. In a preferred embodiment, the vertebrate Smoothened is rat Smoothened (rSmo) or human Smoothened (hSmo). The vertebrate Smoothened may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence vertebrate Smoothened" comprises a protein having the same amino acid sequence as a vertebrate Smoothened derived from nature. Thus, a native sequence vertebrate Smoothened can have the amino acid sequence of naturally occurring human Smoothened, rat Smoothened, or Smoothened from any other vertebrate. Such native sequence vertebrate Smoothened can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence vertebrate Smoothened" specifically encompasses naturally-occurring truncated forms of the vertebrate Smoothened, naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants of the vertebrate Smoothened. In one embodiment of the invention, the native sequence vertebrate Smoothened is a mature native sequence Smoothened comprising the amino acid sequence of SEQ ID NO:4. In another embodiment of the invention, the native sequence vertebrate Smoothened is a mature native sequence Smoothened comprising the amino acid sequence of SEQ ID NO:2.

"Vertebrate Smoothened variant" means a vertebrate Smoothened as defined below having less than 100% sequence identity with vertebrate Smoothened having the deduced amino acid sequence shown in SEQ ID NO:4 for human Smoothened or SEQ ID NO:2 for rat Smoothened. Such vertebrate Smoothened variants include, for instance, vertebrate Smoothened proteins wherein one or more amino acid residues are added at the N- or C-terminus of, or within, the sequences of SEQ ID NO:4 or SEQ ID NO:2; wherein about one to thirty amino acid residues are deleted, or optionally substituted by one or more amino acid residues; and derivatives thereof, wherein an amino acid residue has been covalently modified so that the resulting product has a non-naturally occurring amino acid. Ordinarily, a vertebrate Smoothened variant will have at least about 80% sequence identity, more preferably at least about 90% sequence identity, and even more preferably at least about 95% sequence identity with the sequence of SEQ ID NO:4 or SEQ ID NO:2.

The term "epitope tag" when used herein refers to a tag polypeptide having enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with activity of the vertebrate Smoothened. The tag polypeptide preferably also is fairly unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8-50 amino acid residues (preferably between about 9-30 residues).

"Isolated," when used to describe the various proteins disclosed herein, means protein that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the protein, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous substances. In preferred embodiments, the protein will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated protein includes protein *in situ* within recombinant cells, since at least one component of

the vSmo natural environment will not be present. Ordinarily, however, isolated protein will be prepared by at least one purification step.

An "isolated" vSmo nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the vSmo nucleic acid. An isolated vSmo nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated vSmo nucleic acid molecules therefore are distinguished from the vSmo nucleic acid molecule as it exists in natural cells. However, an isolated vSmo nucleic acid molecule includes vSmo nucleic acid molecules contained in cells that ordinarily express vSmo where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-vSmo monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-vSmo antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-vSmo antibody with a constant domain (*e.g.* "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (*e.g.*, Fab, $F(ab')_2$, and Fv), so long as they exhibit the desired activity. See, *e.g.* U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (*e.g.* murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

The term "vertebrate" as used herein refers to any animal classified as a vertebrate including certain classes of fish, reptiles, birds, and mammals. The term "mammal" as used herein refers to any animal classified as a mammal, including humans, cows, rats, mice, horses, dogs and cats.

II. Modes For Carrying Out The Invention

The present invention is based on the discovery of vertebrate homologues of Smoothened. In particular, Applicants have identified and isolated human and rat Smoothened. The properties and characteristics of human and rat Smoothened are described in further detail in the Examples below. Based upon the properties and characteristics of human and rat Smoothened disclosed herein, it is Applicants' present belief that vertebrate Smoothened is a signalling component in a multi-subunit Hedgehog (particularly Sonic Hedgehog "SHH") receptor.

A description follows as to how vertebrate Smoothened may be prepared.

A. Preparation of vSmo

Techniques suitable for the production of vSmo are well known in the art and include isolating vSmo from an endogenous source of the polypeptide, peptide synthesis (using a peptide synthesizer)

and recombinant techniques (or any combination of these techniques). The description below relates primarily to production of vSmo by culturing cells transformed or transfected with a vector containing vSmo nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare vSmo.

1. Isolation of DNA Encoding vSmo

The DNA encoding vSmo may be obtained from any cDNA library prepared from tissue believed to possess the vSmo mRNA and to express it at a detectable level. Accordingly, human Smo DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the library of human embryonic lung cDNA described in Example 3. Rat Smo DNA can be conveniently obtained from a cDNA library prepared from rat tissues, such as described in Example 1. The vSmo-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the vSmo or oligonucleotides or polypeptides as described in the Examples) designed to identify the gene of interest or the protein encoded by it. The probes are preferably labeled such that they can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P-labeled ATP, biotinylation or enzyme labeling. Screening the cDNA or genomic library with a selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding vSmo is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer:A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequences disclosed herein, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

vSmo variants can be prepared by introducing appropriate nucleotide changes into the vSmo DNA, or by synthesis of the desired vSmo polypeptide. Those skilled in the art will appreciate that amino acid changes (compared to native sequence vSmo) may alter post-translational processes of the vSmo, such as changing the number or position of glycosylation sites.

Variations in the native sequence vSmo can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis.

2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding vSmo may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

(i) Signal Sequence Component

The vSmo may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous amino acid sequence or polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the vSmo DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses.

Most expression vectors are "shuttle" vectors, *i.e.*, they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of vSmo DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the vSmo nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely

adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes vSmo. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding vSmo.

(iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the vSmo nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the vSmo nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, *e.g.*, the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to vSmo encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector.

Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)].

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase,

phosphoglucose isomerase, and glucokinase. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

vSmo transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human β-interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon β1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the vSmo by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981]) and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983]) to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also typically contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain

nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding vSmo.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

(viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding vSmo may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired properties.

(ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of vSmo in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast may be suitable cloning or expression hosts for vSmo-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated vSmo are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells.

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, *e.g.*, Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human

embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture. Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for vSmo production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

4. Culturing the Host Cells

Prokaryotic cells used to produce vSmo may be cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce vSmo may be cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such

as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly ^{32}P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence vSmo protein or against a synthetic peptide based on the DNA sequences provided herein.

6. Purification of vSmo

It is contemplated that it may be desired to purify some form of vSmo from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to vSmo. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. vSmo thereafter may be purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse

phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE: ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG. vSmo variants may be recovered in the same fashion as native sequence vSmo, taking account of any substantial changes in properties occasioned by the variation.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants.

7. Covalent Modifications of vSmo

Covalent modifications of vSmo are included within the scope of this invention. One type of covalent modification of the vSmo included within the scope of this invention comprises altering the native glycosylation pattern of the protein. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence vSmo, and/or adding one or more glycosylation sites that are not present in the native sequence vSmo.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the vSmo may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence vSmo (for O-linked glycosylation sites). The vSmo amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the vSmo protein at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the vSmo is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the vSmo protein may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the

compound trifluoromethanesulfonic acid. or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin. et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

8. vSmo Chimeras

The present invention also provides chimeric molecules comprising vSmo fused to another, heterologous amino acid sequence or polypeptide. In one embodiment, the chimeric molecule comprises a fusion of the vSmo with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally provided at the amino- or carboxyl- terminus of the vSmo. Such epitope-tagged forms of the vSmo are desirable as the presence thereof can be detected using a labeled antibody against the tag polypeptide. Also, provision of the epitope tag enables the vSmo to be readily purified by affinity purification using the anti-tag antibody. Affinity purification techniques and diagnostic assays involving antibodies are described later herein.

Tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides have been disclosed. Examples include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α-tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

The general methods suitable for the construction and production of epitope-tagged vSmo are the same as those disclosed hereinabove. vSmo-tag polypeptide fusions are most conveniently constructed by fusing the cDNA sequence encoding the vSmo portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the vSmo-tag polypeptide chimeras of the present invention, nucleic acid encoding the vSmo will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible.

9. Methods of Using vSmo

vSmo, as disclosed in the present specification, has utility in therapeutic and non-therapeutic applications. As a therapeutic, vSmo (or the nucleic acid encoding the same) can be employed in *in vivo* or *ex vivo* gene therapy techniques. In non-therapeutic applications, nucleic acid sequences encoding the vSmo may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization,

Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding vSmo is present in the cell type(s) being evaluated. vSmo nucleic acid will also be useful for the preparation of vSmo by the recombinant techniques described herein.

The isolated vSmo may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of vSmo may be prepared. vSmo preparations are also useful in generating antibodies, as standards in assays for vSmo (*e.g.*, by labeling vSmo for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), and in affinity purification techniques.

Nucleic acids which encode vSmo, such as the rat vSmo disclosed herein, can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, rat cDNA encoding rSmo or an appropriate sequence thereof can be used to clone genomic DNA encoding Smo in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Smo. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for vSmo transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding vSmo introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding vSmo. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with constitutive activity of vSmo or Hedgehog, including some forms of cancer that may result therefrom, such as for example, basal cell carcinoma, basal cell nevus syndrome and pancreatic carcinoma. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Alternatively, the non-human homologues of vSmo can be used to construct a vSmo "knock out" animal which has a defective or altered gene encoding vSmo as a result of homologous recombination between the endogenous gene encoding vSmo and altered genomic DNA encoding vSmo introduced into an embryonic cell of the animal. For example, rat cDNA encoding Smo can be used to clone genomic DNA encoding Smo in accordance with established techniques. A portion of the genomic DNA encoding Smo can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., Cell, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem*

Cells: A Practical Approach, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and can be used in the study of the mechanism by which the Hedgehog family of molecules exerts mitogenic, differentiative, and morphogenic effects.

B. Anti-vSmo Antibody Preparation

The present invention further provides anti-vSmo antibodies. Antibodies against vSmo may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The vSmo antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the vSmo protein or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

2. Monoclonal Antibodies

The vSmo antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the vSmo protein or a fusion protein thereof. Cells expressing vSmo at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse

myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against vSmo. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an

antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH_1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH_1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab')_2$ antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

3. Humanized Antibodies

The vSmo antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl.

Acad. Sci. USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Bruggermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the vSmo, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

6. Uses of vSmo Antibodies

vSmo antibodies may be used in diagnostic assays for vSmo, *e.g.*, detecting its expression in specific cells or tissues. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

vSmo antibodies also are useful for the affinity detection or purification of vSmo from recombinant cell culture or natural sources. In this process, the antibodies against vSmo are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the vSmo, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the vSmo, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the vSmo from the antibody.

The vSmo antibodies may also be employed as therapeutics. For example, vSmo antibodies may be used to block or neutralize excess vSmo signalling that may result from mutant or inactivated Patched. Accordingly, the vSmo antibodies may be used in the treatment of, or amelioration of symptoms caused by, a pathological condition resulting from or associated with excess vSmo or vSmo signalling. Optionally, agonistic vSmo antibodies can be employed to induce the formation of, or enhance or stimulate tissue regeneration, such as regeneration of skin tissue, lung tissue, muscle (such as heart or skeletal muscle), neural tissue (such as serotonergic neurons, motoneurons or straital neurons), bone tissue or gut tissue. This vSmo antibody therapy will be useful in instances where the tissue has been damaged by disease, aging or trauma.

The vSmo antibodies may be used or administered to a patient in a pharmaceutically-acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo et al. If the vSmo antibodies are to be administered to a patient, the antibodies can be administered by injection (e.g., intravenous, intraperitoneal, subcutaneous, intramuscular), or by other methods such as infusion that ensure its delivery to the bloodstream in an effective form. Effective dosages and schedules for administering the vSmo antibodies may be determined empirically, and making such determinations is within the skill in the art. Those skilled in the art will understand that the dosage of vSmo antibodies that must be administered will vary depending on, for example, the patient which will receive the antibodies, the route of administration, and other therapeutic agents being administered to the mammal. Guidance in selecting appropriate doses for such vSmo antibodies is found in the literature on therapeutic uses of antibodies, e.g., Handbook of Monoclonal Antibodies, Ferrone et al., eds., Noges Publications, Park Ridge, N.J., (1985) ch. 22 and pp. 303-357; Smith et al., Antibodies in Human Diagnosis and Therapy, Haber et al., eds., Raven Press, New York (1977) pp. 365-389. A typical daily dosage of the vSmo antibodies used alone might range from about 1 μg/kg to up to 100 mg/kg of body weight or more per day, depending on the factors mentioned above.

C. Kits Containing vSmo or vSmo Antibodies

In another embodiment of the invention, there are provided articles of manufacture and kits containing vSmo or vSmo antibodies. The article of manufacture typically comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds the vSmo or vSmo antibodies. The label on the container may indicate directions for either *in vivo* or *in vitro* use, such as those described above.

The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, and package inserts with instructions for use.

D. Additional Compositions of Matter

In a further embodiment of the invention, there are provided protein complexes comprising vertebrate Smoothened protein and vertebrate Patched protein. As demonstrated in the Examples, vertebrate Smoothened and vertebrate Patched can form a complex. The protein complex which includes vertebrate Smoothened and vertebrate Patched may also include vertebrate Hedgehog protein. Typically in such a complex, the vertebrate Hedgehog binds to the vertebrate Patched but does not bind to the vertebrate Smoothened. In a preferred embodiment, the complex comprising vertebrate Smoothened and vertebrate Patched is a receptor for vertebrate Hedgehog.

The invention also provides a vertebrate Patched which binds to vertebrate Smoothened. Optionally the vertebrate Patched comprises a sequence which is a derivative of or fragment of a native sequence vertebrate Patched. The vertebrate Patched will typically consist of a sequence which has less than 100% sequence identity with a native sequence vertebrate Patched. In one embodiment, the vertebrate Patched directly and specifically binds vertebrate Smoothened. Alternatively, it is contemplated that the vertebrate Patched may bind vertebrate Smoothened indirectly.

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The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

All commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

EXAMPLE 1

Isolation and Cloning of Rat Smoothened cDNA

Full-length rat Smoothened cDNA was isolated by low stringency hybridization screening of 1.2 x 10^6 plaques of an embryonic day 9-10 rat cDNA library (containing cDNAs size-selected >1500 base pairs), using the entire coding region of Drosophila Smoothened [Alcedo et. al., supra] (labeled with ^{32}P-dCTP) as a probe. The library was prepared by cloning cDNA inserts into the NotI site of a lambda RK18 vector [Klein et. al., Proc. Natl. Acad. Sci., 93:7108-7113 (1996)] following XmnI adapters ligation. Conditions for hybridization were: 5 x SSC, 30% formamide, 5 x Denhardt's, 50 mM sodium phosphate (pH 6.5), 5% dextran sulfate, 0.1% SDS and 50 μg/ml salmon sperm DNA, overnight at 42°C. Nitrocellulose filters were washed to a stringency of 1 x SSC at 42°C, and exposed overnight to Kodak X-AR film. Three of eight positive plaques were selected for further purification. After amplification of the plaque-purified phage, phagemid excision products were generated by growing M13 helper phage (M13K07; obtained from New England Biolabs), bacteria (BB4; obtained from Stratagene), and the purified phage together in a 100:10:1 ratio. Plasmid DNA was recovered by Qiagen purification from ampicillin-resistant colonies following infection of BB4 with the excised purified phagemid.

Sequencing of the three cDNAs showed them to be identical, with the exception that two contained only a partial coding sequence, whereas the third contained the entire open reading frame of rat Smoothened, including 449 and 1022 nucleotides, respectively of 5' and 3' untranslated sequence and a poly-A tail. This cDNA clone was sequenced completely on both strands.

The entire nucleotide sequence of rat Smoothened (rSmo) is shown in Figure 1 (SEQ ID NO:1) (reference is also made to Applicants' ATCC deposit of the rat Smoothened in pRK5.rsmo.AR140, assigned ATCC Dep. No. 98165). The cDNA contained an open reading frame with a translational initiation site assigned to the ATG codon at nucleotide positions 450-452. The open reading frame ends at the termination codon at nucleotide positions 2829-2831.

The predicted amino acid sequence of the rat Smoothened (rSmo) contains 793 amino acids (including a 32 amino acid signal peptide), as shown in Figure 1 (SEQ ID NO:2). rSmo appears to be a typical seven transmembrane (7 TM), G protein-coupled receptor, containing 4 potential N-glycosylation sites and a 203 amino acid long putative extracellular amino-terminus domain which contains 13 stereotypically spaced cysteines (see Fig. 2).

An alignment of the rSmo sequence with sequences for dSmo, wingless receptor and vertebrate Frizzled revealed that rSmo is 33% homologous to the dSmo sequence reported in Alcedo et al., supra (50% homologous in the transmembrane domains); 23% homologous to the wingless receptor sequence reported in Bhanot et al., supra; and 25% homologous to the vertebrate Frizzled sequence reported in Chan et al., supra.

EXAMPLE 2

In Situ Hybridization and Northern Blot Analysis

In situ hybridization and Northern blot analyses were conducted to examine tissue distribution of Smo, Patched and SHH in embryonic and adult rat tissues.

For *in situ* hybridization, E9-E15.5 rat embryos (Hollister Labs) were immersion-fixed overnight at 4°C in 4% paraformaldehyde, then cryoprotected overnight in 20% sucrose. Adult rat brains and spinal cords were frozen fresh. All tissues were sectioned at 16 um, and processed for *in situ* hybridization using ^{33}P-UTP labelled RNA probes as described in Treanor et al., Nature, 382:80-83 (1996). Sense and antisense probes were derived from the N-terminal region of rSmo using T7 polymerase. The probe used to detect SHH was antisense to bases 604-1314 of mouse SHH [Echelard et al., Cell, 75:1417-1430 (1993)]. The probe used to detect Patched was antisense to bases 502-1236 of mouse Patched [Goodrich et al., supra]. Reverse transcriptase polymerase chain reaction analysis was performed as described in Treanor et al., supra.

For Northern blot analysis, a rat multiple tissue Northern blot (Clontech) was hybridized and washed at high stringency according to the manufacturer's protocol, using a ^{32}P-dCTP-labelled probe encompassing the entire rSmo coding region.

The results are illustrated in Figure 3. By *in situ* hybridization and Northern blot analysis, expression of rSmo mRNA was detected from E9 onward in SHH responsive tissues such as the neural folds and early neural tube [Echelard et al., supra, Krauss et al., supra); Roelink et al., supra], pre-somitic mesoderm and somites (Johnson et al., supra; Fan et al., supra], and developing limb buds [Riddle et al., supra] gut (Roberts et al., supra] and eye [Krauss et al., supra]. Rat Smo transcripts were also found in tissues whose development is regulated by other members of the vertebrate HH protein family such as testes (desert HH) [Bitgood et al., Curr. Biol., 6:298-304 (1996)], cartilage (indian HH) [Vortkamp et al., Science, 273:613-622 (1996)], and muscle (the zebra fish, *echinida* HH) [Currie and Ingham, Nature, 382:452-455 (1996)] (See e.g., Fig. 3; other data not shown). In all of the above recited tissues, rSmo appeared to be co-expressed with rPatched.

rSmo and rPatched mRNAs were also found in and around SHH expressing cells in the embryonic lung, epiglottis, thymus, vertebral column, tongue, jaw, taste buds and teeth (Fig. 3). In the embryonic nervous system, rSmo and rPatched are initially expressed throughout the neural plate: by E12, however, their expression declines in lateral parts of the neural tube, and by P1, was restricted to cells in relatively close proximity to the ventricular zone (Fig. 3). In the adult rat tissues, rSmo expression was maintained in the brain, lung, kidney, testis, heart and spleen (data not shown).

EXAMPLE 3

Isolation and Cloning of Human Smoothened cDNA

A cDNA probe corresponding to the coding region of the rat *smoothened* gene (described in Example 1 above) was labeled by the random hexanucleotide method and used to screen 10^6 clones of a human embryonic lung cDNA library (Clontech, Inc.) in lgt10. Duplicate filters were hybridized at 42°C in 50% formamide, 5x SSC, 10x Denhardt's, 0.05M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50 mg/ml of sonicated salmon sperm DNA. Filters were rinsed in 2x SSC and then washed once in 0.5x SSC, 0.1% SDS at 42°C. Hybridizing phage were plaque-purified and the cDNA inserts were subcloned into pUC 118 (New England Biolabs). Two clones, 5 and 14, had overlapping inserts of approximately 2 and 2.8 kb respectively, covering the entire human Smoothened coding sequence (See Fig. 4). Clones 5 and 14 have been deposited by Applicants with ATCC as puc.118.hsmo.5 and puc.118.hsmo.14, respectively, and assigned ATCC Dep. Nos. 98162 and 98163, respectively. Both strands were sequenced by standard fluorescent methods on an ABI377 automated sequencer.

The entire nucleotide sequence of human Smoothened is shown in Figure 4 (SEQ ID NO:3). The cDNA contained an open reading frame with a translational initiation site assigned to the ATG codon at nucleotide positions 13-15. The open reading frame ends at the termination codon at nucleotide positions 2374-2376.

The predicted amino acid sequence of the human Smoothened (hSmo) contains 787 amino acids (including a 29 amino acid signal peptide), as shown in Figure 4 (SEQ ID NO:4). hSmo appears to be a typical seven transmembrane (7 TM), G protein-coupled receptor, containing 5 potential N-glycosylation sites and a 202 amino acid long putative extracellular amino-terminus domain which contains 13 stereotypically spaced cysteines.

An alignment of the predicted hSmo amino acid sequence and rSmo sequence (see Example 1) revealed 94% amino acid identity. An alignment of the hSmo sequence with sequences for dSmo, wingless receptor and vertebrate Frizzled revealed that hSmo is 33% homologous to the dSmo sequence reported in Alcedo et al., supra (50% homologous in the transmembrane domains); 23% homologous to the wingless receptor sequence reported in Bhanot et al., supra; and 25% homologous to the vertebrate Frizzled sequence reported in Chan et al., supra. See Figure 5 for a comparison of the primary sequences of human Smo, rat Smo and Drosophila Smo.

EXAMPLE 4

Competitive binding, Co-immunoprecipitation, and Cross-linking Assays

Competitive binding, co-immunoprecipitation and cross-linking assays were conducted to characterize physical association or binding between SHH and rSmo, and between certain biologically active forms of SHH and cells expressing rSmo, mPatched, or both rSmo and mPatched.

1. Materials and Methods

Complementary DNAs for rSmo (described in Example 1); dSmo (described in Alcedo et al., supra); Desert HH (described in Echelard et al., supra); and murine Patched (described in Goodrich et al., supra) were cloned into pRK5 vectors, and epitope tags [Flag epitope tag (Kodak/IBI) and Myc epitope tag (9E10 epitope: InVitrogen)] added to the extreme C-terminus by PCR-based mutagenesis.

SHH-N is the biologically active amino terminus portion of SHH [Lee et al., Science, 266:1528-1537 (1994)]. SHH-N was produced as described by Hynes et al., supra. A radiolabeled form of SHH-N. ^{125}ISHH-N. was employed.

For IgG-SHH-N production, human embryonic kidney 293 cells were transiently transfected with the expression vector encoding SHH-N fused in frame after amino acid residue 198 to the Fc portion of human IgG-gamma1.

Cells were maintained in serum-free media (OptiMEM: Gibco BRL) for 48 hours. The media was then collected and concentrated 10-fold using a centricon-10 membrane. Conditioned media was used at a concentration of 2x.

Binding assays were conducted to test binding between cells expressing rSmo or dSmo and (1) epitope tagged SHH-N, (2) an IgG-SHH-N chimera, and (3) an epitope tagged Desert HH.

For visualization of SHH binding, COS-7 cells (Genentech, Inc.) transiently expressing rSmo or mPatched (murine Patched) were exposed to epitope tagged SHH-N (2 hours at 4°C), washed 4 times with PBS, then fixed and stained with a cy3-conjugated anti-human IgG (Jackson ImmunoResearch) (for IgG-SHH-N) or anti-Flag M2 antibody (Kodak/IBI) (for Flag-tagged SHH-N).

For immunohistochemistry, COS-7 cells transiently transfected with expression constructs were fixed (10 minutes in 2% paraformaldehyde/0.2% Triton-X 100) and stained using monoclonal anti-Flag M2 antibody (IBI) or anti-Myc antibody (InVitrogen), followed by cy3-conjugated anti-mouse IgG (Jackson Immunoresearch).

For cross-linking, cells were resuspended at a density of 1-2 x 10^6/ml in ice-cold L15 media containing 0.1% BSA and 50 pM ^{125}I-labeled SHH (with or without a 1000-fold excess of unlabeled SHH) and incubated at 4°C for 2 hr. 10 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodimide HCl and 5 mM N-hydroxysulfosuccinimide (Pierce Chemical) were added to the samples and incubated at room temperature for 30 minutes. The cells were then washed 3 times with 1 ml of PBS. Cells were then lysed in lysis buffer [1% Brij-96 (Sigma), 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM PMSF, 10 µM aprotinin, 10 µM leupeptin] and the protein complexes were immunoprecipitated with antibodies to the epitope tags as indicated. Immunoprecipitated proteins were resuspended in sample buffer (80 mM Tris-HCl [pH 6.8], 10% [v/v] glycerol, 1% [w/v] SDS, 0.025% Bromphenol Blue, denatured and run on 4% SDS-polyacrylamide gels, which were dried and exposed to film.

For the equilibrium binding analysis, the cells were processed as above, and incubated with 50 pM ^{125}I-SHH and various concentrations of cold SHH-N (Cold Ligand). The IGOR program was used to determine K_d.

2. Results

The results are shown in Figure 6. No binding of epitope tagged SHH-N, of IgG-SHH-N chimeric protein or of an epitope tagged Desert HH to cells expressing rSmo or dSmo was observed (Figures 6a-b and data not shown). This data (and the data described below) indicated that rSmo, acting alone, would not likely be a receptor for SHH or Desert HH. However, it was hypothesized that rSmo is a component in a multi-subunit SHH receptor complex and that the ligand binding function of this receptor complex would be provided by another membrane protein such as Patched.

Binding assays were also conducted to test binding between cells expressing rSmo or murine patched and (1) an epitope tagged SHH and (2) an IgG-SHH-N chimera. The data shows that epitope tagged SHH-N as well as an IgG-SHH-N chimeric protein bind specifically and reversibly to cells expressing the mouse Patched (mPatched) (mPatched is 33% identical to Drosophila Patched) (Figure. 6c-e). Furthermore, only mPatched could be immunoprecipitated by the IgG-SHH-N protein (Fig. 6f) and antibodies to an epitope tagged mPatched readily co-immunoprecipitated ^{125}I-SHH-N (Fig. 6h) (antibodies to epitope tagged rSmo could not immunoprecipitate ^{125}I-SHH-N and the IgG-SHH-N chimera did not immunoprecipitate rSmo).

As shown in Fig. 6g, the cross-linking assay of ^{125}I-SHH-N to cells expressing rSmo or mPatched in the presence or absence of cold SHH-N revealed that ^{125}I-SHH-N is cross-linked only to mPatched expressing cells.

The competitive binding assay of ^{125}I-SHH-N and cells expressing mPatched or mPatched plus rSmo also showed that mPatched and SHH-N had a relatively high affinity of interaction (approximate K_d of 460 pM) (Fig. 6i). This corresponds well to the concentrations of SHH-N which are required to elicit biological responses in multiple systems [Fan et al., supra; Hynes et al. supra; Roelink et al., supra]. No binding to cells expressing rSmo alone was observed (data not shown) and there was no increase in binding affinity to mPatched in the presence of rSmo.

EXAMPLE 5

Co-immunoprecipitation Assays

To determine whether Patched and Smo form or interact in a physical complex, co-immunoprecipitation experiments were performed.

1. Materials and Methods

For the double immunohistochemistry, COS-7 cells transiently transfected with expression constructs were permeabilized using 0.2% Triton-x 100. The cells were fixed (10 minutes in 2% paraformaldehyde/0.2% Triton-X 100) and stained using monoclonal anti-Flag M2 antibody (IBI) and rabbit polyclonal anti-Myc primary antibodies (Santa Cruz Biotech), followed by cy3-conjugated anti-mouse IgG (Jackson Immunoresearch) and bodipy-conjugated anti-rabbit IgG secondary antibodies (Molecular Probes, Inc.).

Human embryonic kidney 293 cells were transiently transfected with expression vectors for epitope tagged rSmo (Flag epitope) and mPatched (Myc epitope) and the resulting proteins complexes were immunoprecipitated with antibody to one of the epitopes and then analyzed on a western blot.

For the co-immunoprecipitation assay, lysates from 293 embryonic kidney cells transiently expressing Flag-tagged rSmo, Myc-tagged mPatched or a combination of the two proteins were incubated (48 hours after transfection) in the presence or absence of the IgG-SHH-N chimera (1 μg/ml, 30 minutes at 37°C) or in the presence of ^{125}I-SHH-N with or without an excess of cold SHH-N (2 hours at 4°C). The incubated samples were then washed 3 times with PBS, and lysed in lysis buffer (see Example 4) as described by Davis et al., Science, 259:1736-1739 (1993). The cell lysates were centrifuged at 10,000 rpm for 10 minutes, and the soluble protein complexes were immunoprecipitated with either protein A sepharose (for the IgG-SHH-N), or anti-Flag or anti-Myc antibodies followed by protein A sepharose (for the epitope-tagged rSmo or mPatched, respectively).

The samples were heated to 100°C for 5 minutes in denaturing SDS sample buffer (125 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 100 mM b-mercaptoethanol, 0.05% bromphenol blue) and subjected to SDS-PAGE. The proteins were detected either by exposure of the dried gel to film (for ^{125}I-SHH-N) or by blotting to nitrocellulose and probing with antibodies to Flag or Myc epitopes using the ECL detection system (Amersham).

2. Results

The results are illustrated in Figure 7. In cells expressing mPatched alone, or rSmo alone, no co-immunoprecipitated protein complexes could be detected. In contrast, in cells that expressed both mPatched and rSmo (Fig. 7a), rSmo was readily co-immunoprecipitated by antibodies to the epitope tagged mPatched (Fig. 7b) and mPatched was co-immunoprecipitated by antibodies to the epitope tagged rSmo (Fig. 7c).

The ^{125}I-SHH-N was readily co-immunoprecipitated by antibodies to the epitope tagged rSmo or mPatched from cells that expressed both rSmo and mPatched, but not from cells expressing rSmo alone (Figs. 7d and 7e). These results indicate that SHH-N, rSmo and mPatched are present in the same physical complex, and that a rSmo-SHH complex does not form in the absence of mPatched. Although not fully understood and not being bound by any particular theory, it is believed that Patched is a ligand binding component and vSmo is a signalling component in a multi-subunit SHH receptor (See, Fig. 9). Patched is also believed to be a negative regulator of vSmo.

EXAMPLE 6

Hahn et al., supra, Johnson et al., supra, and Gailani et al., supra, report that Patched mutations have been associated with BCNS and sporadic basal cell carcinoma ("BCC"). These investigators also report that most of the Patched mutations in BCNS are truncations in which no functional protein is produced. It is believed that BCNS and BCC may be caused or associated with constitutive activation of vSmo, following its release from negative regulation by Patched.

Expression levels of wild-type (native) murine Patched and a mutant Patched were examined. A Patched mutant was generated by site-directed mutagenesis of the wild-type mouse Patched cDNA (described in Example 4) and verified by sequencing. The mutant Patched contained a 3 amino acid insertion (Pro-Asn-Ile) after amino acid residue 815 (this mutant was found in a BCNS family, see, Hahn et al., supra). For analysis of protein expression, equal amounts of pRK5 expression vectors containing wild-type or mutant Patched were transfected into 293 cells, and an equal number of cells (2×10^6) were lysed per sample. Proteins were immunoprecipitated from cell lysates by antibody to the Patched epitope tag (myc) and detected on a Western blot with the same antibody.

Applicants found that expression of the mutant Patched (which retains a complete open reading frame) was reduced at least 10-fold as compared to its wild-type counterpart. See Fig. 8.

* * * * *

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Material	ATCC Dep. No.	Deposit Date
puc.118.hsmo.5	98162	Sept. 6, 1996
puc.118.hsmo.14	98163	Sept. 6, 1996
pRK5.rsmo.AR140	98165	Sept. 10, 1996

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Genentech, Inc.

(ii) TITLE OF INVENTION: Vertebrate Smoothened Proteins

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
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(B) STREET: 460 Point San Bruno Blvd
(C) CITY: South San Francisco
(D) STATE: California
(E) COUNTRY: USA
(F) ZIP: 94080

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WinPatin (Genentech)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Svoboda, Craig G.
(B) REGISTRATION NUMBER: 39,044
(C) REFERENCE/DOCKET NUMBER: P1050PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 415/225-1489
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3854 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATCGGGGCCC CAGAGGGTTC CCAGGGTTGA AGACAATTCC AATCGAGGCG 200

AGGGAGTCCG GGGTCCGTGC ATCCTGGCCC GGGCCTGCGC AGCTCAACAT 250

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Thr Gly Pro Gly Pro Arg Ser Ala Gly Gly Ser Ala Arg
 40                  45                  50

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         55                  60                  65

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Ser His Cys Gly Arg Ala Ala His Cys Glu Pro Leu Arg
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Tyr Asn Val Cys Leu Gly Ser Ala Leu Pro Tyr Gly Ala
     80                  85                  90

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Thr Thr Thr Leu Leu Ala Gly Asp Ser Asp Ser Gln Glu
             95                  100

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Glu Ala His Ser Lys Leu Val Leu Trp Ser Gly Leu Arg
105                 110                 115

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Asn Ala Pro Arg Cys Trp Ala Val Ile Gln Pro Leu Leu
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        575                 580                 585

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AGCTTCAGCC AGGCAAGAAA GCTTCTTCCA ACTTCTGTAT CTGGTGGGAA 3680

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CAGAGGCTCA GTATGAGAAG AAGAAATATG AACAGTAAAT AAAACATTTT 3830

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 793 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Arg Leu Leu Gln Leu Leu Leu Leu Val Leu Leu Gly Gly Arg Gly
                20                  25                  30

Arg Gly Ala Ala Leu Ser Gly Asn Val Thr Gly Pro Gly Pro Arg
                35                  40                  45

Ser Ala Gly Gly Ser Ala Arg Arg Asn Ala Pro Val Thr Ser Pro
                50                  55                  60

Pro Pro Pro Leu Leu Ser His Cys Gly Arg Ala Ala His Cys Glu
                65                  70                  75

Pro Leu Arg Tyr Asn Val Cys Leu Gly Ser Ala Leu Pro Tyr Gly
                80                  85                  90

Ala Thr Thr Thr Leu Leu Ala Gly Asp Ser Asp Ser Gln Glu Glu
                95                  100                 105

Ala His Ser Lys Leu Val Leu Trp Ser Gly Leu Arg Asn Ala Pro
                110                 115                 120

Arg Cys Trp Ala Val Ile Gln Pro Leu Leu Cys Ala Val Tyr Met
                125                 130                 135

Pro Lys Cys Glu Asn Asp Arg Val Glu Leu Pro Ser Arg Thr Leu
                140                 145                 150

Cys Gln Ala Thr Arg Gly Pro Cys Ala Ile Val Glu Arg Glu Arg
                155                 160                 165

Gly Trp Pro Asp Phe Leu Arg Cys Thr Pro Asp His Phe Pro Glu
                170                 175                 180

Gly Cys Pro Asn Glu Val Gln Asn Ile Lys Phe Asn Ser Ser Gly
                185                 190                 195

Gln Cys Glu Ala Pro Leu Val Arg Thr Asp Asn Pro Lys Ser Trp
                200                 205                 210

Tyr Glu Asp Val Glu Gly Cys Gly Ile Gln Cys Gln Asn Pro Leu
                215                 220                 225
```

Phe Thr Glu Ala Glu His Gln Asp Met His Ser Tyr Ile Ala Ala
230 235 240

Phe Gly Ala Val Thr Gly Leu Cys Thr Leu Phe Thr Leu Ala Thr
245 250 255

Phe Val Ala Asp Trp Arg Asn Ser Asn Arg Tyr Pro Ala Val Ile
260 265 270

Leu Phe Tyr Val Asn Ala Cys Phe Phe Val Gly Ser Ile Gly Trp
275 280 285

Leu Ala Gln Phe Met Asp Gly Ala Arg Arg Glu Ile Val Cys Arg
290 295 300

Ala Asp Gly Thr Met Arg Phe Gly Glu Pro Thr Ser Ser Glu Thr
305 310 315

Leu Ser Cys Val Ile Ile Phe Val Ile Val Tyr Tyr Ala Leu Met
320 325 330

Ala Gly Val Val Trp Phe Val Val Leu Thr Tyr Ala Trp His Thr
335 340 345

Ser Phe Lys Ala Leu Gly Thr Thr Tyr Gln Pro Leu Ser Gly Lys
350 355 360

Thr Ser Tyr Phe His Leu Leu Thr Trp Ser Leu Pro Phe Val Leu
365 370 375

Thr Val Ala Ile Leu Ala Val Ala Gln Val Asp Gly Asp Ser Val
380 385 390

Ser Gly Ile Cys Phe Val Gly Tyr Lys Asn Tyr Arg Tyr Arg Ala
395 400 405

Gly Phe Val Leu Ala Pro Ile Gly Leu Val Leu Ile Val Gly Gly
410 415 420

Tyr Phe Leu Ile Arg Gly Val Met Thr Leu Phe Ser Ile Lys Ser
425 430 435

Asn His Pro Gly Leu Leu Ser Glu Lys Ala Ala Ser Lys Ile Asn
440 445 450

Glu Thr Met Leu Arg Leu Gly Ile Phe Gly Phe Leu Ala Phe Gly
455 460 465

Phe Val Leu Ile Thr Phe Ser Cys His Phe Tyr Asp Phe Phe Asn
470 475 480

Gln Ala Glu Trp Glu Arg Ser Phe Arg Asp Tyr Val Leu Cys Gln
485 490 495

Ala Asn Val Thr Ile Gly Leu Pro Thr Lys Lys Pro Ile Pro Asp
500 505 510

Cys Glu Ile Lys Asn Arg Pro Ser Leu Leu Val Glu Lys Ile Asn

515 520 525

Leu Phe Ala Met Phe Gly Thr Gly Ile Ala Met Ser Thr Trp Val
530 535 540

Trp Thr Lys Ala Thr Leu Leu Ile Trp Arg Arg Thr Trp Cys Arg
545 550 555

Leu Thr Gly His Ser Asp Asp Glu Pro Lys Arg Ile Lys Lys Ser
560 565 570

Lys Met Ile Ala Lys Ala Phe Ser Lys Arg Arg Glu Leu Leu Gln
575 580 585

Asn Pro Gly Gln Glu Leu Ser Phe Ser Met His Thr Val Ser His
590 595 600

Asp Gly Pro Val Ala Gly Leu Ala Phe Glu Leu Asn Glu Pro Ser
605 610 615

Ala Asp Val Ser Ser Ala Trp Ala Gln His Val Thr Lys Met Val
620 625 630

Ala Arg Arg Gly Ala Ile Leu Pro Gln Asp Val Ser Val Thr Pro
635 640 645

Val Ala Thr Pro Val Pro Pro Glu Glu Gln Ala Asn Leu Trp Leu
650 655 660

Val Glu Ala Glu Ile Ser Pro Glu Leu Glu Lys Arg Leu Gly Arg
665 670 675

Lys Lys Lys Arg Arg Lys Arg Lys Lys Glu Val Cys Pro Leu Gly
680 685 690

Pro Ala Pro Glu Leu His His Ser Ala Pro Val Pro Ala Thr Ser
695 700 705

Ala Val Pro Arg Leu Pro Gln Leu Pro Arg Gln Lys Cys Leu Val
710 715 720

Ala Ala Asn Ala Trp Gly Thr Gly Glu Pro Cys Arg Gln Gly Ala
725 730 735

Trp Thr Val Val Ser Asn Pro Phe Cys Pro Glu Pro Ser Pro His
740 745 750

Gln Asp Pro Phe Leu Pro Gly Ala Ser Ala Pro Arg Val Trp Ala
755 760 765

Gln Gly Arg Leu Gln Gly Leu Gly Ser Ile His Ser Arg Thr Asn
770 775 780

Leu Met Glu Ala Glu Leu Leu Asp Ala Asp Ser Asp Phe
785 790 793

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2972 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CGGGGGTTGG CC   ATG GCC GCT GCC CGC CCA GCG CGG GGG 39
                Met Ala Ala Ala Arg Pro Ala Arg Gly
                  1               5

CCG GAG CTC CCG CTC CTG GGG CTG CTG CTG CTG CTG CTG 78
Pro Glu Leu Pro Leu Leu Gly Leu Leu Leu Leu Leu Leu
 10                 15                  20

CTG GGG GAC CCG GGC CGG GGG GCG GCC TCG AGC GGG AAC 117
Leu Gly Asp Pro Gly Arg Gly Ala Ala Ser Ser Gly Asn
        25                  30                  35

GCG ACC GGG CCT GGG CCT CGG AGC GCG GGC GGG AGC GCG 156
Ala Thr Gly Pro Gly Pro Arg Ser Ala Gly Gly Ser Ala
                40                  45

AGG AGG AGC GCG GCG GTG ACT GGC CCT CCG CCG CCG CTG 195
Arg Arg Ser Ala Ala Val Thr Gly Pro Pro Pro Pro Leu
    50                  55                  60

AGC CAC TGC GGC CGG GCT GCC CCC TGC GAG CCG CTG CGC 234
Ser His Cys Gly Arg Ala Ala Pro Cys Glu Pro Leu Arg
            65                  70

TAC AAC GTG TGC CTG GGC TCG GTG CTG CCC TAC GGG GCC 273
Tyr Asn Val Cys Leu Gly Ser Val Leu Pro Tyr Gly Ala
 75                 80                  85

ACC TCC ACA CTG CTG GCC GGA GAC TCG GAC TCC CAG GAG 312
Thr Ser Thr Leu Leu Ala Gly Asp Ser Asp Ser Gln Glu
        90                  95                 100

GAA GCG CAC GGC AAG CTC GTG CTC TGG TCG GGC CTC CGG 351
Glu Ala His Gly Lys Leu Val Leu Trp Ser Gly Leu Arg
                105                 110

AAT GCC CCC CGC TGC TGG GCA GTG ATC CAG CCC CTG CTG 390
Asn Ala Pro Arg Cys Trp Ala Val Ile Gln Pro Leu Leu
    115                 120                 125

TGT GCC GTA TAC ATG CCC AAG TGT GAG AAT GAC CGG GTG 429
Cys Ala Val Tyr Met Pro Lys Cys Glu Asn Asp Arg Val
            130                 135

GAG CTG CCC AGC CGT ACC CTC TGC CAG GCC ACC CGA GGC 468
Glu Leu Pro Ser Arg Thr Leu Cys Gln Ala Thr Arg Gly
140                 145                 150

CCC TGT GCC ATC GTG GAG AGG GAG CGG GGC TGG CCT GAC 507
Pro Cys Ala Ile Val Glu Arg Glu Arg Gly Trp Pro Asp
```

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        155                 160                 165

TTC CTG CGC TGC ACT CCT GAC CGC TTC CCT GAA GGC TGC 546
Phe Leu Arg Cys Thr Pro Asp Arg Phe Pro Glu Gly Cys
                170                 175

ACG AAT GAG GTG CAG AAC ATC AAG TTC AAC AGT TCA GGC 585
Thr Asn Glu Val Gln Asn Ile Lys Phe Asn Ser Ser Gly
    180                 185                 190

CAG TGC GAA GTG CCC TTG GTT CGG ACA GAC AAC CCC AAG 624
Gln Cys Glu Val Pro Leu Val Arg Thr Asp Asn Pro Lys
            195                 200

AGC TGG TAC GAG GAC GTG GAG GGC TGC GGC ATC CAG TGC 663
Ser Trp Tyr Glu Asp Val Glu Gly Cys Gly Ile Gln Cys
205                 210                 215

CAG AAC CCG CTC TTC ACA GAG GCT GAG CAC CAG GAC ATG 702
Gln Asn Pro Leu Phe Thr Glu Ala Glu His Gln Asp Met
        220                 225                 230

CAC AGC TAC ATC GCG GCC TTC GGG GCC GTC ACG GGC CTC 741
His Ser Tyr Ile Ala Ala Phe Gly Ala Val Thr Gly Leu
                235                 240

TGC ACG CTC TTC ACC CTG GCC ACA TTC GTG GCT GAC TGG 780
Cys Thr Leu Phe Thr Leu Ala Thr Phe Val Ala Asp Trp
    245                 250                 255

CGG AAC TCG AAT CGC TAC CCT GCT GTT ATT CTC TTC TAC 819
Arg Asn Ser Asn Arg Tyr Pro Ala Val Ile Leu Phe Tyr
            260                 265

GTC AAT GCG TGC TTC TTT GTG GGC AGC ATT GGC TGG CTG 858
Val Asn Ala Cys Phe Phe Val Gly Ser Ile Gly Trp Leu
270                 275                 280

GCC CAG TTC ATG GAT GGT GCC CGC CGA GAG ATC GTC TGC 897
Ala Gln Phe Met Asp Gly Ala Arg Arg Glu Ile Val Cys
        285                 290                 295

CGT GCA GAT GGC ACC ATG AGG CTT GGG GAG CCC ACC TCC 936
Arg Ala Asp Gly Thr Met Arg Leu Gly Glu Pro Thr Ser
                300                 305

AAT GAG ACT CTG TCC TGC GTC ATC ATC TTT GTC ATC GTG 975
Asn Glu Thr Leu Ser Cys Val Ile Ile Phe Val Ile Val
    310                 315                 320

TAC TAC GCC CTG ATG GCT GGT GTG GTT TGG TTT GTG GTC 1014
Tyr Tyr Ala Leu Met Ala Gly Val Val Trp Phe Val Val
            325                 330

CTC ACC TAT GCC TGG CAC ACT TCC TTC AAA GCC CTG GGC 1053
Leu Thr Tyr Ala Trp His Thr Ser Phe Lys Ala Leu Gly
335                 340                 345
```

```
ACC ACC TAC CAG CCT CTC TCG GGC AAG ACC TCC TAC TTC 1092
Thr Thr Tyr Gln Pro Leu Ser Gly Lys Thr Ser Tyr Phe
        350                 355                 360

CAC CTG CTC ACC TGG TCA CTC CCC TTT GTC CTC ACT GTG 1131
His Leu Leu Thr Trp Ser Leu Pro Phe Val Leu Thr Val
                365                 370

GCA ATC CTT GCT GTG GCG CAG GTG GAT GGG GAC TCT GTG 1170
Ala Ile Leu Ala Val Ala Gln Val Asp Gly Asp Ser Val
    375                 380                 385

AGT GGC ATT TGT TTT GTG GGC TAC AAG AAC TAC CGA TAC 1209
Ser Gly Ile Cys Phe Val Gly Tyr Lys Asn Tyr Arg Tyr
            390                 395

CGT GCG GGC TTC GTG CTG GCC CCA ATC GGC CTG GTG CTC 1248
Arg Ala Gly Phe Val Leu Ala Pro Ile Gly Leu Val Leu
400                 405                 410

ATC GTG GGA GGC TAC TTC CTC ATC CGA GGA GTC ATG ACT 1287
Ile Val Gly Gly Tyr Phe Leu Ile Arg Gly Val Met Thr
        415                 420                 425

CTG TTC TCC ATC AAG AGC AAC CAC CCC GGG CTG CTG AGT 1326
Leu Phe Ser Ile Lys Ser Asn His Pro Gly Leu Leu Ser
                430                 435

GAG AAG GCT GCC AGC AAG ATC AAC GAG ACC ATG CTG CGC 1365
Glu Lys Ala Ala Ser Lys Ile Asn Glu Thr Met Leu Arg
    440                 445                 450

CTG GGC ATT TTT GGC TTC CTG GCC TTT GGC TTT GTG CTC 1404
Leu Gly Ile Phe Gly Phe Leu Ala Phe Gly Phe Val Leu
            455                 460

ATT ACC TTC AGC TGC CAC TTC TAC GAC TTC TTC AAC CAG 1443
Ile Thr Phe Ser Cys His Phe Tyr Asp Phe Phe Asn Gln
465                 470                 475

GCT GAG TGG GAG CGC AGC TTC CGG GAC TAT GTG CTA TGT 1482
Ala Glu Trp Glu Arg Ser Phe Arg Asp Tyr Val Leu Cys
        480                 485                 490

CAG GCC AAT GTG ACC ATC GGG CTG CCC ACC AAG CAG CCC 1521
Gln Ala Asn Val Thr Ile Gly Leu Pro Thr Lys Gln Pro
                495                 500

ATC CCT GAC TGT GAG ATC AAG AAT CGC CCG AGC CTT CTG 1560
Ile Pro Asp Cys Glu Ile Lys Asn Arg Pro Ser Leu Leu
    505                 510                 515

GTG GAG AAG ATC AAC CTG TTT GCC ATG TTT GGA ACT GGC 1599
Val Glu Lys Ile Asn Leu Phe Ala Met Phe Gly Thr Gly
            520                 525

ATC GCC ATG AGC ACC TGG GTC TGG ACC AAG GCC ACG CTG 1638
Ile Ala Met Ser Thr Trp Val Trp Thr Lys Ala Thr Leu
```

530 535 540

CTC ATC TGG AGG CGT ACC TGG TGC AGG TTG ACT GGG CAG 1677
Leu Ile Trp Arg Arg Thr Trp Cys Arg Leu Thr Gly Gln
545 550 555

AGT GAC GAT GAG CCA AAG CGG ATC AAG AAG AGC AAG ATG 1716
Ser Asp Asp Glu Pro Lys Arg Ile Lys Lys Ser Lys Met
560 565

ATT GCC AAG GCC TTC TCT AAG CGG CAC GAG CTC CTG CAG 1755
Ile Ala Lys Ala Phe Ser Lys Arg His Glu Leu Leu Gln
570 575 580

AAC CCA GGC CAG GAG CTG TCC TTC AGC ATG CAC ACT GTG 1794
Asn Pro Gly Gln Glu Leu Ser Phe Ser Met His Thr Val
585 590

TCC CAC GAC GGG CCC GTG GCG GGC TTG GCC TTT GAC CTC 1833
Ser His Asp Gly Pro Val Ala Gly Leu Ala Phe Asp Leu
595 600 605

AAT GAG CCC TCA GCT GAT GTC TCC TCT GCC TGG GCC CAG 1872
Asn Glu Pro Ser Ala Asp Val Ser Ser Ala Trp Ala Gln
610 615 620

CAT GTC ACC AAG ATG GTG GCT CGG AGA GGA GCC ATA CTG 1911
His Val Thr Lys Met Val Ala Arg Arg Gly Ala Ile Leu
625 630

CCC CAG GAT ATT TCT GTC ACC CCT GTG GCA ACT CCA GTG 1950
Pro Gln Asp Ile Ser Val Thr Pro Val Ala Thr Pro Val
635 640 645

CCC CCA GAG GAA CAA GCC AAC CTG TGG CTG GTT GAG GCA 1989
Pro Pro Glu Glu Gln Ala Asn Leu Trp Leu Val Glu Ala
650 655

GAG ATC TCC CCA GAG CTG CAG AAG CGC CTG GGC CGG AAG 2028
Glu Ile Ser Pro Glu Leu Gln Lys Arg Leu Gly Arg Lys
660 665 670

AAG AAG AGG AGG AAG AGG AAG AAG GAG GTG TGC CCG CTG 2067
Lys Lys Arg Arg Lys Arg Lys Lys Glu Val Cys Pro Leu
675 680 685

GCG CCG CCC CCT GAG CTT CAC CCC CCT GCC CCT GCC CCC 2106
Ala Pro Pro Pro Glu Leu His Pro Pro Ala Pro Ala Pro
690 695

AGT ACC ATT CCT CGA CTG CCT CAG CTG CCC CGG CAG AAA 2145
Ser Thr Ile Pro Arg Leu Pro Gln Leu Pro Arg Gln Lys
700 705 710

TGC CTG GTG GCT GCA GGT GCC TGG GGA GCT GGG GAC TCT 2184
Cys Leu Val Ala Ala Gly Ala Trp Gly Ala Gly Asp Ser
715 720

```
TGC CGA CAG GGA GCG TGG ACC CTG GTC TCC AAC CCA TTC 2223
Cys Arg Gln Gly Ala Trp Thr Leu Val Ser Asn Pro Phe
725                 730                 735

TGC CCA GAG CCC AGT CCC CCT CAG GAT CCA TTT CTG CCC 2262
Cys Pro Glu Pro Ser Pro Pro Gln Asp Pro Phe Leu Pro
        740                 745                 750

AGT GCA CCG GCC CCC GTG GCA TGG GCT CAT GGC CGC CGA 2301
Ser Ala Pro Ala Pro Val Ala Trp Ala His Gly Arg Arg
                755                 760

CAG GGC CTG GGG CCT ATT CAC TCC CGC ACC AAC CTG ATG 2340
Gln Gly Leu Gly Pro Ile His Ser Arg Thr Asn Leu Met
    765                 770                 775

GAC ACA GAA CTC ATG GAT GCA GAC TCG GAC TTC TGAGCCT 2380
Asp Thr Glu Leu Met Asp Ala Asp Ser Asp Phe
            780                 785     787

GCAGAGCAGG ACCTGGGACA GGAAAGAGAG GAACCAATAC CTTCAAGGCT 2430

CTTCTTCCTC ACCGAGCATG CTTCCCTAGG ATCCCGTCTT CCAGAGAACC 2480

TGTGGGCTGA CTGCCCTCCG AAGAGAGTTC TGGATGTCTG GCTCAAAGCA 2530

GCAGGACTGT GGGAAAGAGC CTAACATCTC CATGGGGAGG CCTCACCCCA 2580

GGGACAGGGC CCTGGAGCTC AGGGTCCTTG TTTCTGCCCT GCCAGCTGCA 2630

GCCTGGTTGG CAGCATCTGC TCCATCGGGG CAGGGGGTAT GCAGAGCTTG 2680

TGGTGGGGCA GGAACGGTGG AGGCAGAGGT GACAGTTCCC AGAGTGGGCT 2730

TTGGTGGCCA GGGAGGCAGC CTAGCCTATG TCTGGCAGAT GAGGGCTGGC 2780

TGCCGTTTTC TGGGCTGATG GGTGCCCTTT CCTGGCAGTC TCAGTCCAAA 2830

AGTGTTGACT GTGTCATTAG TCCTTTGTCT AAGTAGGGCC AGGGCACCGT 2880

ATTCCTCTCC CAGGTGTTTG TGGGGCTGGA AGGACCTGCT CCCACAGGGG 2930

CCATGTCCTC TCTTAATAGG TGGCACTACC CCAAACCCAC CG 2972
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 787 amino acids
(B) TYPE: Amino Acid
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Ala Ala Ala Arg Pro Ala Arg Gly Pro Glu Leu Pro Leu Leu
 1               5                  10                  15

Gly Leu Leu Leu Leu Leu Leu Leu Gly Asp Pro Gly Arg Gly Ala
                20                  25                  30
```

```
Ala Ser Ser Gly Asn Ala Thr Gly Pro Gly Pro Arg Ser Ala Gly
                35                  40                  45

Gly Ser Ala Arg Arg Ser Ala Ala Val Thr Gly Pro Pro Pro Pro
                50                  55                  60

Leu Ser His Cys Gly Arg Ala Ala Pro Cys Glu Pro Leu Arg Tyr
                65                  70                  75

Asn Val Cys Leu Gly Ser Val Leu Pro Tyr Gly Ala Thr Ser Thr
                80                  85                  90

Leu Leu Ala Gly Asp Ser Asp Ser Gln Glu Glu Ala His Gly Lys
                95                  100                 105

Leu Val Leu Trp Ser Gly Leu Arg Asn Ala Pro Arg Cys Trp Ala
                110                 115                 120

Val Ile Gln Pro Leu Leu Cys Ala Val Tyr Met Pro Lys Cys Glu
                125                 130                 135

Asn Asp Arg Val Glu Leu Pro Ser Arg Thr Leu Cys Gln Ala Thr
                140                 145                 150

Arg Gly Pro Cys Ala Ile Val Glu Arg Glu Arg Gly Trp Pro Asp
                155                 160                 165

Phe Leu Arg Cys Thr Pro Asp Arg Phe Pro Glu Gly Cys Thr Asn
                170                 175                 180

Glu Val Gln Asn Ile Lys Phe Asn Ser Ser Gly Gln Cys Glu Val
                185                 190                 195

Pro Leu Val Arg Thr Asp Asn Pro Lys Ser Trp Tyr Glu Asp Val
                200                 205                 210

Glu Gly Cys Gly Ile Gln Cys Gln Asn Pro Leu Phe Thr Glu Ala
                215                 220                 225

Glu His Gln Asp Met His Ser Tyr Ile Ala Ala Phe Gly Ala Val
                230                 235                 240

Thr Gly Leu Cys Thr Leu Phe Thr Leu Ala Thr Phe Val Ala Asp
                245                 250                 255

Trp Arg Asn Ser Asn Arg Tyr Pro Ala Val Ile Leu Phe Tyr Val
                260                 265                 270

Asn Ala Cys Phe Phe Val Gly Ser Ile Gly Trp Leu Ala Gln Phe
                275                 280                 285

Met Asp Gly Ala Arg Arg Glu Ile Val Cys Arg Ala Asp Gly Thr
                290                 295                 300

Met Arg Leu Gly Glu Pro Thr Ser Asn Glu Thr Leu Ser Cys Val
                305                 310                 315

Ile Ile Phe Val Ile Val Tyr Tyr Ala Leu Met Ala Gly Val Val
```

320 325 330

Trp Phe Val Val Leu Thr Tyr Ala Trp His Thr Ser Phe Lys Ala
335 340 345

Leu Gly Thr Thr Tyr Gln Pro Leu Ser Gly Lys Thr Ser Tyr Phe
350 355 360

His Leu Leu Thr Trp Ser Leu Pro Phe Val Leu Thr Val Ala Ile
365 370 375

Leu Ala Val Ala Gln Val Asp Gly Asp Ser Val Ser Gly Ile Cys
380 385 390

Phe Val Gly Tyr Lys Asn Tyr Arg Tyr Arg Ala Gly Phe Val Leu
395 400 405

Ala Pro Ile Gly Leu Val Leu Ile Val Gly Gly Tyr Phe Leu Ile
410 415 420

Arg Gly Val Met Thr Leu Phe Ser Ile Lys Ser Asn His Pro Gly
425 430 435

Leu Leu Ser Glu Lys Ala Ala Ser Lys Ile Asn Glu Thr Met Leu
440 445 450

Arg Leu Gly Ile Phe Gly Phe Leu Ala Phe Gly Phe Val Leu Ile
455 460 465

Thr Phe Ser Cys His Phe Tyr Asp Phe Phe Asn Gln Ala Glu Trp
470 475 480

Glu Arg Ser Phe Arg Asp Tyr Val Leu Cys Gln Ala Asn Val Thr
485 490 495

Ile Gly Leu Pro Thr Lys Gln Pro Ile Pro Asp Cys Glu Ile Lys
500 505 510

Asn Arg Pro Ser Leu Leu Val Glu Lys Ile Asn Leu Phe Ala Met
515 520 525

Phe Gly Thr Gly Ile Ala Met Ser Thr Trp Val Trp Thr Lys Ala
530 535 540

Thr Leu Leu Ile Trp Arg Arg Thr Trp Cys Arg Leu Thr Gly Gln
545 550 555

Ser Asp Asp Glu Pro Lys Arg Ile Lys Lys Ser Lys Met Ile Ala
560 565 570

Lys Ala Phe Ser Lys Arg His Glu Leu Leu Gln Asn Pro Gly Gln
575 580 585

Glu Leu Ser Phe Ser Met His Thr Val Ser His Asp Gly Pro Val
590 595 600

Ala Gly Leu Ala Phe Asp Leu Asn Glu Pro Ser Ala Asp Val Ser
605 610 615

```
Ser Ala Trp Ala Gln His Val Thr Lys Met Val Ala Arg Arg Gly
                620                 625                 630

Ala Ile Leu Pro Gln Asp Ile Ser Val Thr Pro Val Ala Thr Pro
                635                 640                 645

Val Pro Pro Glu Glu Gln Ala Asn Leu Trp Leu Val Glu Ala Glu
                650                 655                 660

Ile Ser Pro Glu Leu Gln Lys Arg Leu Gly Arg Lys Lys Lys Arg
                665                 670                 675

Arg Lys Arg Lys Lys Glu Val Cys Pro Leu Ala Pro Pro Pro Glu
                680                 685                 690

Leu His Pro Pro Ala Pro Ala Pro Ser Thr Ile Pro Arg Leu Pro
                695                 700                 705

Gln Leu Pro Arg Gln Lys Cys Leu Val Ala Ala Gly Ala Trp Gly
                710                 715                 720

Ala Gly Asp Ser Cys Arg Gln Gly Ala Trp Thr Leu Val Ser Asn
                725                 730                 735

Pro Phe Cys Pro Glu Pro Ser Pro Pro Gln Asp Pro Phe Leu Pro
                740                 745                 750

Ser Ala Pro Ala Pro Val Ala Trp Ala His Gly Arg Arg Gln Gly
                755                 760                 765

Leu Gly Pro Ile His Ser Arg Thr Asn Leu Met Asp Thr Glu Leu
                770                 775                 780

Met Asp Ala Asp Ser Asp Phe
                785         787
```

WHAT IS CLAIMED IS:

1. Isolated vertebrate Smoothened.

2. Isolated vertebrate Smoothened having at least about 80% sequence identity with native sequence vertebrate Smoothened comprising amino acid residues 1 to 787 of SEQ ID NO:4.

3. The vertebrate Smoothened of claim 2 wherein said Smoothened has at least about 90% sequence identity.

4. The vertebrate Smoothened of claim 3 wherein said Smoothened has at least about 95% sequence identity.

5. Isolated native sequence vertebrate Smoothened comprising the amino acid sequence of SEQ ID NO:4.

6. Isolated native sequence vertebrate Smoothened comprising the amino acid sequence of SEQ ID NO:2.

7. A chimeric molecule comprising the vertebrate Smoothened of claim 1 fused to a heterologous amino acid sequence.

8. The chimeric molecule of claim 7 wherein said heterologous amino acid sequence is an epitope tag sequence.

9. An antibody which specifically binds to the vertebrate Smoothened of claim 1.

10. The antibody of claim 9 wherein said antibody is a monoclonal antibody.

11. The antibody of claim 9 which is a neutralizing antibody.

12. The antibody of claim 9 which is an agonist antibody.

13. Isolated nucleic acid encoding vertebrate Smoothened.

14. The nucleic acid of claim 13 wherein said nucleic acid encodes native sequence vertebrate Smoothened comprising the amino acid sequence of SEQ ID NO:4.

15. The nucleic acid of claim 13 wherein said nucleic acid encodes native sequence vertebrate Smoothened comprising the amino acid sequence of SEQ ID NO:2.

16. A vector comprising the nucleic acid of claim 13.

17. The vector of claim 16 operably linked to control sequences recognized by a host cell transformed with the vector.

18. A host cell comprising the vector of claim 16.

19. A process of using a nucleic acid molecule encoding vertebrate Smoothened to effect production of vertebrate Smoothened comprising culturing the host cell of claim 18.

20. The process of claim 19 further comprising recovering the vertebrate Smoothened from the host cell culture.

21. An article of manufacture, comprising a container and a composition contained within said container, wherein the composition includes vertebrate Smoothened or vertebrate Smoothened antibodies.

22. The article of manufacture of claim 21 further comprising instructions for using the vertebrate Smoothened or vertebrate Smoothened antibodies *in vivo* or *ex vivo*.

23. A non-human, transgenic animal which contains cells that express nucleic acid encoding vertebrate Smoothened.

24. The animal of claim 23 which is a mouse or rat.

25. A non-human, knockout animal which contains cells having an altered gene encoding vertebrate Smoothened.

26. The animal of claim 25 which is a mouse or rat.

27. A protein complex comprising vertebrate Smoothened protein and vertebrate Patched protein.

28. The protein complex of claim 27 further comprising vertebrate Hedgehog protein.

29. The protein complex of claim 28 wherein the vertebrate Hedgehog protein binds to the vertebrate Patched protein but does not bind to the vertebrate Smoothened protein.

30. The protein complex of claim 27 which is a receptor for vertebrate Hedgehog protein.

31. A vertebrate Patched which binds to vertebrate Smoothened.

32. The vertebrate Patched of claim 31 which has less than 100% sequence identity with a native sequence vertebrate Patched.

```
GCGGCGCGCT CGCGCGGAGG TGGCTGCTGG GCCGCGGGCT GGCGTGGGGG 50

CGGAGCCGGG GAGCGACTCC CGCACCCCAC GGCCGGTGCC TGCCCTCCAT 100

CGAGGGGCTG GGAGTTAGTT TTAATGGTGG GAGAGGGAAT GGGGCTGAAG 150

ATCGGGGCCC CAGAGGGTTC CCAGGGTTGA AGACAATTCC AATCGAGGCG 200

AGGGAGTCCG GGGTCCGTGC ATCCTGGCCC GGGCCTGCGC AGCTCAACAT 250

GGGGCCCGGG TTCCAAAGTT TGCAAAGTTG GGAGCCGAGG GGCCCGGACG 300

CGCGCGGCGC CTGGCGAAAG CTGGCCCCAG ACTTTCGGGG CGCACCGGTC 350

GCCTAAGTAG CCTCCGCGGC CCCCGGGGTC GTGTGTGTGG CCAGGGGACT 400

CCGGGGAGCT CGGGGGCGCC TCAGCTTCTG CTGAGTTGGC GGTTTGGCC  449

ATG GCT GCT GGC CGC CCC GTG CGT GGG CCC GAG CTG GCG 488
Met Ala Ala Gly Arg Pro Val Arg Gly Pro Glu Leu Ala
 1               5                  10

CCC CGG AGG CTG CTG CAG TTG CTG CTG CTG GTA CTG CTT 527
Pro Arg Arg Leu Leu Gln Leu Leu Leu Leu Val Leu Leu
    15                  20                  25

GGG GGC CGG GGC CGG GGG GCG GCC TTG AGC GGG AAC GTG 566
Gly Gly Arg Gly Arg Gly Ala Ala Leu Ser Gly Asn Val
            30                  35

ACC GGG CCT GGG CCT CGC AGT GCC GGC GGG AGC GCG AGG 605
Thr Gly Pro Gly Pro Arg Ser Ala Gly Gly Ser Ala Arg
40                  45                  50

AGG AAC GCG CCG GTG ACC AGC CCT CCG CCG CCG CTG CTG 644
Arg Asn Ala Pro Val Thr Ser Pro Pro Pro Pro Leu Leu
        55                  60                  65

AGC CAC TGC GGC CGG GCC GCC CAC TGC GAG CCT TTG CGC 683
Ser His Cys Gly Arg Ala Ala His Cys Glu Pro Leu Arg
                70                  75

TAC AAC GTG TGC CTG GGC TCC GCG CTG CCC TAC GGA GCC 722
Tyr Asn Val Cys Leu Gly Ser Ala Leu Pro Tyr Gly Ala
    80                  85                  90

ACC ACC ACG CTG CTG GCT GGG GAC TCG GAC TCG CAG GAG 761
Thr Thr Thr Leu Leu Ala Gly Asp Ser Asp Ser Gln Glu
            95                  100

GAA GCG CAC AGC AAG CTC GTG CTC TGG TCC GGC CTC CGG 800
Glu Ala His Ser Lys Leu Val Leu Trp Ser Gly Leu Arg
105                 110                 115
```

FIG._1A

```
AAT GCT CCC CGA TGC TGG GCA GTG ATC CAG CCC CTG CTG 839
Asn Ala Pro Arg Cys Trp Ala Val Ile Gln Pro Leu Leu
        120                 125                 130

TGT GCT GTC TAC ATG CCC AAG TGT GAA AAT GAC CGA GTG 878
Cys Ala Val Tyr Met Pro Lys Cys Glu Asn Asp Arg Val
                135                 140

GAG TTG CCC AGC CGT ACC CTC TGC CAG GCC ACC CGA GGC 917
Glu Leu Pro Ser Arg Thr Leu Cys Gln Ala Thr Arg Gly
    145                 150                 155

CCC TGT GCC ATT GTG GAG CGG GAA CGA GGG TGG CCT GAC 956
Pro Cys Ala Ile Val Glu Arg Glu Arg Gly Trp Pro Asp
            160                 165

TTT CTG CGT TGC ACG CCG GAC CAC TTC CCT GAA GGC TGT 995
Phe Leu Arg Cys Thr Pro Asp His Phe Pro Glu Gly Cys
170                 175                 180

CCA AAC GAG GTA CAA AAC ATC AAG TTC AAC AGT TCA GGC 1034
Pro Asn Glu Val Gln Asn Ile Lys Phe Asn Ser Ser Gly
        185                 190                 195

CAA TGT GAA GCA CCC TTG GTG AGG ACA GAC AAC CCC AAG 1073
Gln Cys Glu Ala Pro Leu Val Arg Thr Asp Asn Pro Lys
                200                 205

AGC TGG TAC GAG GAC GTG GAG GGC TGT GGG ATC CAG TGC 1112
Ser Trp Tyr Glu Asp Val Glu Gly Cys Gly Ile Gln Cys
    210                 215                 220

CAG AAC CCG CTG TTC ACC GAG GCT GAG CAC CAG GAC ATG 1151
Gln Asn Pro Leu Phe Thr Glu Ala Glu His Gln Asp Met
            225                 230

CAC AGT TAC ATC GCA GCC TTC GGG GCG GTC ACC GGC CTC 1190
His Ser Tyr Ile Ala Ala Phe Gly Ala Val Thr Gly Leu
235                 240                 245

TGT ACA CTC TTC ACC CTG GCC ACC TTT GTG GCT GAC TGG 1229
Cys Thr Leu Phe Thr Leu Ala Thr Phe Val Ala Asp Trp
        250                 255                 260

CGG AAC TCC AAT CGC TAC CCT GCG GTT ATT CTC TTC TAT 1268
Arg Asn Ser Asn Arg Tyr Pro Ala Val Ile Leu Phe Tyr
                265                 270

GTC AAT GCG TGT TTC TTT GTG GGC AGC ATT GGC TGG CTG 1307
Val Asn Ala Cys Phe Ph  Val Gly Ser Ile Gly Trp Leu
    275                 280                 285
```

FIG._1B

```
TAC TAT GCC TTG ATG GCT GGA GTA GTG TGG TTC GTG GTC 1463
Tyr Tyr Ala Leu Met Ala Gly Val Val Trp Phe Val Val
                330                 335

CTC ACC TAT GCC TGG CAC ACC TCC TTC AAA GCC CTG GGC 1502
Leu Thr Tyr Ala Trp His Thr Ser Phe Lys Ala Leu Gly
    340                 345                 350

ACC ACT TAC CAG CCT CTC TCG GGC AAG ACA TCC TAT TTC 1541
Thr Thr Tyr Gln Pro Leu Ser Gly Lys Thr Ser Tyr Phe
            355                 360

CAC CTG CTC ACG TGG TCA CTC CCC TTC GTC CTC ACT GTG 1580
His Leu Leu Thr Trp Ser Leu Pro Phe Val Leu Thr Val
365                 370                 375

GCA ATC CTT GCT GTG GCT CAG GTA GAT GGG GAC TCC GTG 1619
Ala Ile Leu Ala Val Ala Gln Val Asp Gly Asp Ser Val
        380                 385                 390

AGT GGC ATC TGC TTT GTA GGC TAC AAG AAC TAT CGG TAC 1658
Ser Gly Ile Cys Phe Val Gly Tyr Lys Asn Tyr Arg Tyr
                395                 400

CGT GCT GGC TTT GTA CTT GCC CCA ATT GGC CTG GTG CTT 1697
Arg Ala Gly Phe Val Leu Ala Pro Ile Gly Leu Val Leu
    405                 410                 415

ATT GTG GGA GGC TAC TTC CTC ATC CGA GGG GTC ATG ACT 1736
Ile Val Gly Gly Tyr Phe Leu Ile Arg Gly Val Met Thr
            420                 425

CTG TTC TCC ATC AAG AGC AAC CAC CCT GGG CTT CTG AGT 1775
Leu Phe Ser Ile Lys Ser Asn His Pro Gly Leu Leu Ser
430                 435                 440

GAG AAG GCA GCC AGC AAG ATC AAT GAG ACC ATG CTG CGC 1814
Glu Lys Ala Ala Ser Lys Ile Asn Glu Thr Met Leu Arg
        445                 450                 455

CTG GGC ATT TTT GGC TTC CTC GCC TTT GGC TTC GTG CTC 1853
Leu Gly Ile Phe Gly Phe Leu Ala Phe Gly Phe Val Leu
                460                 465

ATC ACC TTC AGC TGC CAC TTC TAT GAC TTC TTC AAC CAG 1892
Ile Thr Phe Ser Cys His Phe Tyr Asp Phe Phe Asn Gln
    470                 475                 480

GCT GAG TGG GAG CGT AGC TTC CGG GAC TAT GTG CTA TGC 1931
Ala Glu Trp Glu Arg Ser Phe Arg Asp Tyr Val Leu Cys
            485                 490
```

FIG._1C

```
CAA GCC AAT GTG ACC ATT GGG CTG CCT ACC AAG AAG CCC 1970
Gln Ala Asn Val Thr Ile Gly Leu Pro Thr Lys Lys Pro
495                 500                 505

ATT CCT GAT TGT GAG ATC AAG AAT CGG CCC AGC CTC CTG 2009
Ile Pro Asp Cys Glu Ile Lys Asn Arg Pro Ser Leu Leu
        510                 515                 520

GTG GAG AAG ATC AAT CTG TTT GCC ATG TTT GGC ACT GGC 2048
Val Glu Lys Ile Asn Leu Phe Ala Met Phe Gly Thr Gly
                525                 530

ATT GCC ATG AGC ACC TGG GTC TGG ACC AAG GCC ACC CTG 2087
Ile Ala Met Ser Thr Trp Val Trp Thr Lys Ala Thr Leu
    535                 540                 545

CTC ATC TGG AGG CGC ACC TGG TGC AGG TTG ACT GGG CAC 2126
Leu Ile Trp Arg Arg Thr Trp Cys Arg Leu Thr Gly His
            550                 555

AGT GAT GAT GAA CCC AAG AGA ATC AAG AAA AGC AAG ATG 2165
Ser Asp Asp Glu Pro Lys Arg Ile Lys Lys Ser Lys Met
560                 565                 570

ATT GCC AAG GCC TTC TCT AAG CGG CGT GAA CTG CTG CAG 2204
Ile Ala Lys Ala Phe Ser Lys Arg Arg Glu Leu Leu Gln
        575                 580                 585

AAC CCG GGC CAG GAG CTC TCC TTC AGC ATG CAC ACT GTC 2243
Asn Pro Gly Gln Glu Leu Ser Phe Ser Met His Thr Val
                590                 595

TCC CAT GAT GGA CCT GTT GCC GGT TTG GCT TTT GAA CTC 2282
Ser His Asp Gly Pro Val Ala Gly Leu Ala Phe Glu Leu
    600                 605                 610

AAT GAA CCC TCA GCT GAT GTC TCC TCT GCC TGG GCC CAG 2321
Asn Glu Pro Ser Ala Asp Val Ser Ser Ala Trp Ala Gln
            615                 620

CAC GTC ACC AAG ATG GTG GCT CGA AGA GGA GCC ATA TTA 2360
His Val Thr Lys Met Val Ala Arg Arg Gly Ala Ile Leu
625                 630                 635

CCC CAG GAT GTG TCT GTC ACC CCT GTG GCA ACT CCA GTG 2399
Pro Gln Asp Val Ser Val Thr Pro Val Ala Thr Pro Val
        640                 645                 650

CCA CCA GAA GAA CAA GCC AAC CTG TGG CTG GTT GAG GCA 2438
Pro Pro Glu Glu Gln Ala Asn Leu Trp Leu Val Glu Ala
                655                 660
```

FIG._1D

```
GAG ATC TCC CCA GAG TTA GAG AAG CGT TTA GGC CGG AAG 2477
Glu Ile Ser Pro Glu Leu Glu Lys Arg Leu Gly Arg Lys
    665                 670                 675

AAG AAG CGG AGG AAG AGG AAG AAG GAG GTG TGC CCC TTG 2516
Lys Lys Arg Arg Lys Arg Lys Lys Glu Val Cys Pro Leu
            680                 685

GGG CCA GCC CCT GAA CTT CAC CAC TCT GCC CCT GTT CCT 2555
Gly Pro Ala Pro Glu Leu His His Ser Ala Pro Val Pro
690                 695                 700

GCC ACC AGT GCA GTT CCT CGG CTG CCT CAG CTG CCT CGG 2594
Ala Thr Ser Ala Val Pro Arg Leu Pro Gln Leu Pro Arg
        705                 710                 715

CAG AAG TGC CTA GTA GCT GCA AAT GCC TGG GGA ACA GGA 2633
Gln Lys Cys Leu Val Ala Ala Asn Ala Trp Gly Thr Gly
                720                 725

GAG CCC TGC CGA CAG GGA GCC TGG ACT GTA GTC TCC AAC 2672
Glu Pro Cys Arg Gln Gly Ala Trp Thr Val Val Ser Asn
    730                 735                 740

CCC TTC TGC CCA GAG CCT AGT CCC CAT CAA GAT CCA TTT 2711
Pro Phe Cys Pro Glu Pro Ser Pro His Gln Asp Pro Phe
            745                 750

CTC CCT GGT GCC TCA GCC CCC AGG GTC TGG GCT CAG GGC 2750
Leu Pro Gly Ala Ser Ala Pro Arg Val Trp Ala Gln Gly
755                 760                 765

CGC CTC CAG GGG CTG GGA TCC ATT CAT TCC CGC ACT AAC 2789
Arg Leu Gln Gly Leu Gly Ser Ile His Ser Arg Thr Asn
        770                 775                 780

CTA ATG GAG GCT GAG CTC TTG GAT GCA GAC TCG GAC TTC TG 2830
Leu Met Glu Ala Glu Leu Leu Asp Ala Asp Ser Asp Phe
                785                 790         793

AGCTTGCAGG GCAGGTCCTA GGATGGGGAA GACAAGTGCA CGCCTTCCTA 2880

TAGCTCTTCC TGAGAGCACA CCTCTGGGGT CTCATCTGAC AGTCTATGGG 2930

CCATGTATCT GCCTACAAGA GCTGTGTACG ACTGGCTAGA AGCAGCCAGA 2980

CCATAGAAAC AAGCTGAACA CAGCCACTGA TAGACCTCAC TTCAGAAGCA 3030

AGACCTGCAG TTCAGGACCC TTGCCTCTGC CCCCCAATTA GAGTCTGGCT 3080

GGCAGTGTTA GTCTCCAACA GAGCTTGTAC TAGGGTAGGA ACGGCAGAGG 3130

CAGGGGTGAT GGTACCCAGA GTGGGCTGGG GTGTCCAGTG AGGTAACCAA 3180
```

FIG._1E

GCCCATGTCT GGCAGATGAG GGCTGGCTGC CCTTTTCTGT GCCAATGAGT 3230

GCCCTTTTCT GGCGCTCTGA GACCAAAAGT GTTTATTGTG TCATTTGTCC 3280

TTTTTCTAGG TGGGAACAGG ACTCTCTTTT TCCTCTTCCT GGTAGTTGTA 3330

ATGACTACTC CCATAAGGCC TAGAACTGCT CTCAGTAGGT GGCCCTGTCC 3380

AAAACACATC TTCACATCTT AGTTCCACTA GGCCAAACTC TTATTGGTTA 3430

GCACCTTAAA ACACACACAC ACACACACAC ACACACACAC ACACACACAC 3480

ACACACACAC ACCCTCTTAC TTCTGAGCTT GGTCTCAAGA GAGAGACAAC 3530

TGGTTCAGCT CCAGGCCTCT GAGAGTCATG TTTTCTTCCT CACATCCATC 3580

CAGTGGGGAT GGATCCTCTG ACTTAAGGGG CTACCTTGGG AAGCCTCTGT 3630

AGCTTCAGCC AGGCAAGAAA GCTTCTTCCA ACTTCTGTAT CTGGTGGGAA 3680

GGAGGACTCC CTACTTTTTA CAATGTCTAG TCATTTTCAT AGTGCCCCAC 3730

ATTCAAGAAC CAGACAGCAG GATGCCTTAG AAGCTGGCTG GGTTCCAGGT 3780

CAGAGGCTCA GTATGAGAAG AAGAAATATG AACAGTAAAT AAAACATTTT 3830

TGTATAAAAA AAAAAAAAAA AAAA 3854

FIG._1F

```
rSmo    1  MAAGRPVRGPELAPRRLLQLLL.LLVLLGGRGRGAALSGNVTGPGPRSAGG
dSmo    1  MQYLNFPRMPNIMMFLEVAILCLWVVADASASSAKFGSTTPASAQQSDVE

rSmo   50  SARRNAPV........TSPPPPLLS.......HCGRAAHCEPLRY..NVC
dSmo   51  LEPINGTLNYRLYAKKGRDDKPWFDGLDSRHIQCVRRARCYPTSNATNTC

rSmo   83  LGSALPYGATTTLLAGDSDSQEEAHSKLVLWSGLRNAPRCWAVIQPLLCA
dSmo  101  FGSKLPYELSSLDLT.DFHTEKELNDKLNDYYALKHVPKCWAAIQPFLCA

rSmo  133  VYMPKCE....NDRVELPSRTLCQATRGPCAIVERERGWPDFLRCTPDHF
dSmo  150  VFKPKCEKINGEDMVYLPSYEMCRITMEPCRILYNTTFFPKFLRCNETLF

rSmo  179  PEGCPNEVQNIKFNSSGQCEAPLVRTDNPKSWYEDVEGCGIQCQNPLFTE
dSmo  200  PTKCTNGARGMKFNGTGQCLSPLVPTDTSASYYPGIEGCGVRCKDPLYTD

rSmo  229  AEHQDMHSYIAAFGAWTGLCVLFTLATFVADWRNSNRYPAVILFYVNACF
dSmo  250  DEHRQIHKLIGWAGSICLLSNLFVVSTFFIDWKNANKYPAVIVFYINLCF

rSmo  279  FVGSIGWLAQFMDGARREIVCRADGTMRFGEPTSSETLSCVIIFVIVYYA
dSmo  300  LIACVGWLLQFTSGSREDIVCRKDGTLRHSEPTAGENLSCIVIFVLVYYF

rSmo  329  LMAGVVWFVVLTYAWHTSFKALGTTYQPLSGKTSYFHLLTWSLPFVLTVA
dSmo  350  LTAGMVWFVFLTYAWH..WRAMGHVQDRIDKKGSYFHLVAWSLPLVLTIT

rSmo  379  ILAVAQVDGDSVSGICFVGYKNYRYRAGFVLAPIGLVLTVGGYFQIRGVM
dSmo  398  TMAFSEVDGNSIVGICFVGYINHSMRAGLLGPLCGVILLGGYFITRGMV

rSmo  429  TLFSIKSNHPGLLSEKAASKINETMLRLGTFGFLAFGFVLITFSCHFYDF
dSmo  448  MLFGLKHFANDIKSTSASNKIHLIIMRMGVCALLTLVFILVAIACHVTEF
```

FIG._2A

```
rSmo  479  F N Q A E W E R S F R D Y V L C Q A N V T I G L P T K K P I P D C E I K N R P S L L V E K I N L F A
dSmo  498  R H A D E W A Q S F R Q F I I C . . K I S S V F E E K . . . S S C R I E N R P S V G V L Q L H L L C

rSmo  529  M F G T G I A M S T W V W T K A T L L I W R R T W C R L T G H S D D E P K R I K K S K M I A K A F S
dSmo  543  L F S S G I V M S T W C W T P S S I E T W K R Y I R K K C G K E V V E E V K M P K H K V I A Q T W A

rSmo  579  K R R E L L Q N P G Q E L S F S M H T V S H D G P V A G L A F E L N E . . . . P S A D V S S A W A Q
dSmo  593  K R K D . F E D K G R . L S I T L Y N . T H T D P V . G L N F D V N D L N S S E T N D I S S T W A A

rSmo  625  H V T K M V A R R . . . . G A I L P Q D V S V T P V A T P V P P E E Q A N L W L . . . . . . . . . V E
dSmo  639  Y L P Q C V K R R M A L T G A A T G N S S S H G P R K N S L D S E I S V S V R H V S V E S R R N S V

rSmo  663  A E I S P E L E K R L G R K K K R R K R K K E V C P L G P A P E L H H S A P V P A T S A V P R L P Q
dSmo  689  D S Q V S V K I A E M K T K V A S R S R G K H G G S S S N R R T Q R R R D Y I A A A T . . G K S S R

rSmo  713  L P R Q K C L V A A N A W G T G E P C R Q G A W T V V S N P F C P E P S P H Q D P F L P G A S A P R
dSmo  737  R R E S S T S V E S Q V I A L K K T T Y P N A S H K V G V F A H H S S K K Q H N Y T S S M K R R T A

rSmo  763  V W A Q G R L Q G L G S I H S R T N L M E A E L L D A D S D F
dSmo  787  N A G L D P S I L N E F L Q K N G D F I F P F L Q N Q D M S S S S E E D N S R A S Q K I Q D L N V V

dSmo  837  V K Q Q E I S E D D H D G I K I E E L P N S K Q V A L E N F L K N I K K S N E S N S N R H S R N S A

dSmo  887  R S Q S K K S Q K R H L K N P A A D L D F R K D C V K Y R S N D S L S C S S E E L D V A L D V G S L

dSmo  937  L N S S F S G I S M G K P H S R N S K T S C D V G I Q A N P F E L V P S Y G E D E L Q Q A M R L L N

dSmo  987  A A S R Q R T E A A N E D F G G T E L Q G L L G H S H R H Q R E P T F M S E S D K L K M L L L P S K
```

FIG._2B

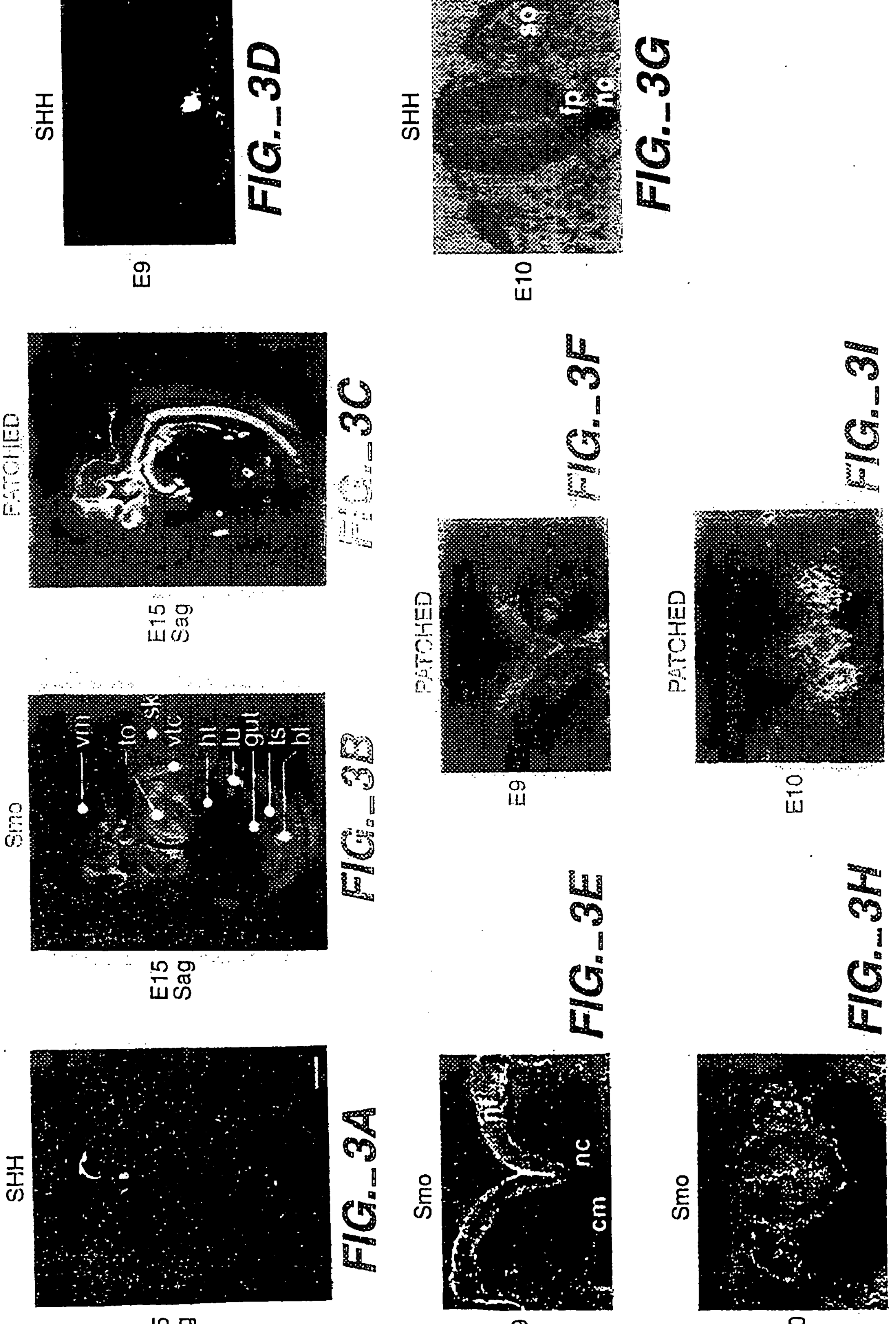


FIG._3A FIG._3B FIG._3C FIG._3D FIG._3E FIG._3F FIG._3G FIG._3H FIG._3I

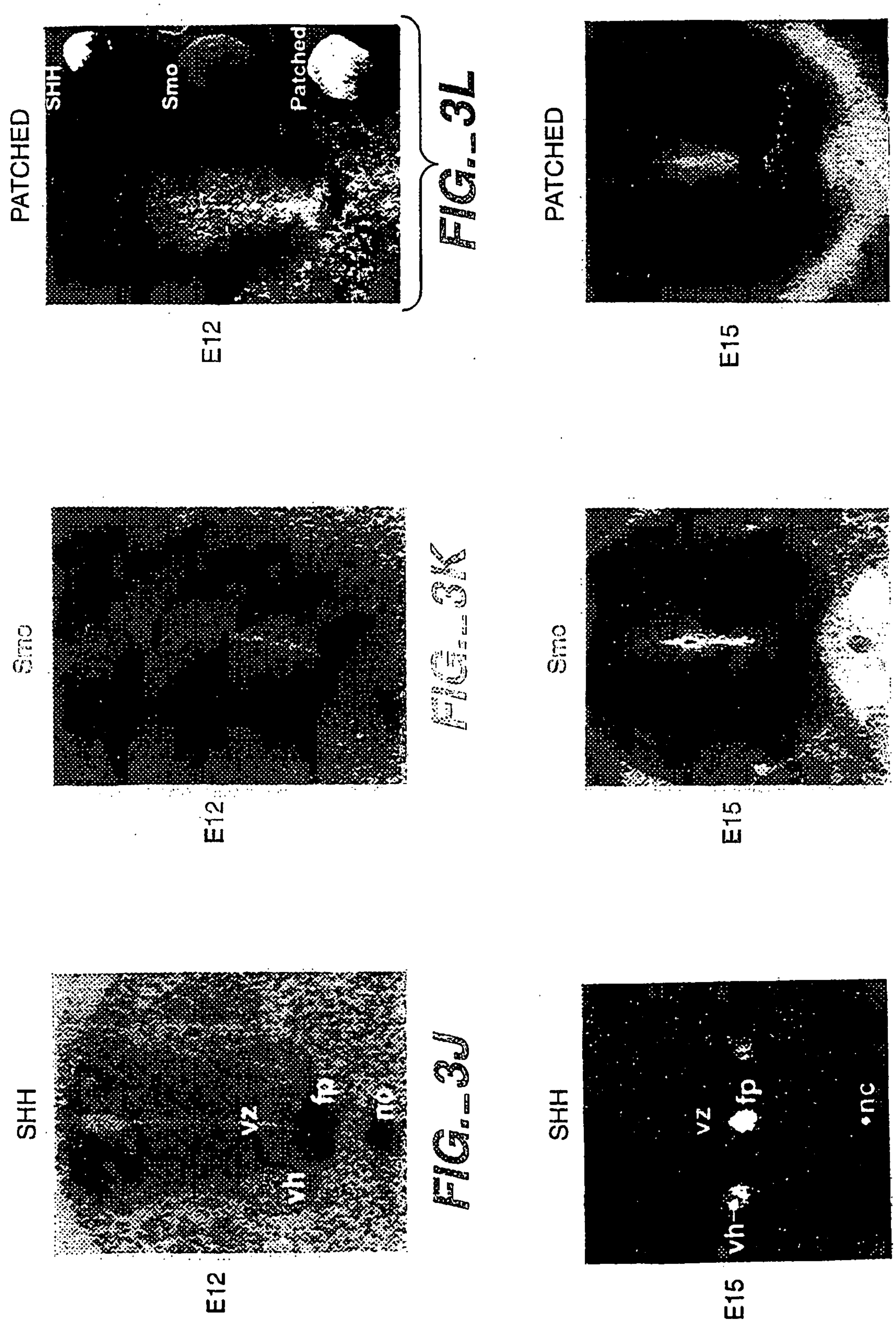


FIG._3L

FIG._3O

FIG._3K

FIG._3N

FIG._3J

FIG._3M

```
  1  CGGGGGTTGG CCATGGCCGC TGCCCGCCCA GCGCGGGGGC CGGAGCTCCC
     GCCCCCAACC GGTACCGGCG ACGGGCGGGT CGCGCCCCCG GCCTCGAGGG
  1             MetAlaAl aAlaArgPro AlaArgGlyP roGluLeuPr
                Met

 51  GCTCCTGGGG CTGCTGCTGC TGCTGCTGCT GGGGGACCCG GGCCGGGGGG
     CGAGGACCCC GACGACGACG ACGACGACGA CCCCCTGGGC CCGGCCCCCC
 14  oLeuLeuGly LeuLeuLeuL euLeuLeuLe uGlyAspPro GlyArgGlyAla

101  CGGCCTCGAG CGGGAACGCG ACCGGGCCTG GGCCTCGGAG CGCGGGCGGG
     GCCGGAGCTC GCCCTTGCGC TGGCCCGGAC CCGGAGCCTC GCGCCCGCCC
 31    AlaSerSe rGlyAsnAla ThrGlyProG lyProArgSe rAlaGlyGly

151  AGCGCGAGGA GGAGCGCGGC GGTGACTGGC CCTCCGCCGC CGCTGAGCCA
     TCGCGCTCCT CCTCGCGCCG CCACTGACCG GGAGGCGGCG GCGACTCGGT
 47  SerAlaArgA rgSerAlaAl aValThrGly ProProProP roLeuSerHis

201  CTGCGGCCGG GCTGCCCCCT GCGAGCCGCT GCGCTACAAC GTGTGCCTGG
     GACGCCGGCC CGACGGGGGA CGCTCGGCGA CGCGATGTTG CACACGGACC
 64   CysGlyArg AlaAlaProC ysGluProLe uArgTyrAsn ValCysLeuG

251  GCTCGGTGCT GCCCTACGGG GCCACCTCCA CACTGCTGGC CGGAGACTCG
     CGAGCCACGA CGGGATGCCC CGGTGGAGGT GTGACGACCG GCCTCTGAGC
 81  lySerValLe uProTyrGly AlaThrSerT hrLeuLeuAl aGlyAspSer

301  GACTCCCAGG AGGAAGCGCA CGGCAAGCTC GTGCTCTGGT CGGGCCTCCG
     CTGAGGGTCC TCCTTCGCGT GCCGTTCGAG CACGAGACCA GCCCGGAGGC
 97  AspSerGlnG luGluAlaHi sGlyLysLeu ValLeuTrpS erGlyLeuAr

351  GAATGCCCCC CGCTGCTGGG CAGTGATCCA GCCCCTGCTG TGTGCCGTAT
     CTTACGGGGG GCGACGACCC GTCACTAGGT CGGGGACGAC ACACGGCATA
114  gAsnAlaPro ArgCysTrpA laValIleGl nProLeuLeu CysAlaValTyr

401  ACATGCCCAA GTGTGAGAAT GACCGGGTGG AGCTGCCCAG CCGTACCCTC
     TGTACGGGTT CACACTCTTA CTGGCCCACC TCGACGGGTC GGCATGGGAG
131    MetProLy sCysGluAsn AspArgValG luLeuProSe rArgThrLeu

451  TGCCAGGCCA CCCGAGGCCC CTGTGCCATC GTGGAGAGGG AGCGGGGCTG
     ACGGTCCGGT GGGCTCCGGG GACACGGTAG CACCTCTCCC TCGCCCCGAC
147  CysGlnAlaT hrArgGlyPr oCysAlaIle ValGluArgG luArgGlyTrp

501  GCCTGACTTC CTGCGCTGCA CTCCTGACCG CTTCCCTGAA GGCTGCACGA
     CGGACTGAAG GACGCGACGT GAGGACTGGC GAAGGGACTT CCGACGTGCT
164   ProAspPhe LeuArgCysT hrProAspAr gPheProGlu GlyCysThrA

551  ATGAGGTGCA GAACATCAAG TTCAACAGTT CAGGCCAGTG CGAAGTGCCC
     TACTCCACGT CTTGTAGTTC AAGTTGTCAA GTCCGGTCAC GCTTCACGGG
181  snGluValGl nAsnIleLys PheAsnSerS erGlyGlnCy sGluValPro

601  TTGGTTCGGA CAGACAACCC CAAGAGCTGG TACGAGGACG TGGAGGGCTG
     AACCAAGCCT GTCTGTTGGG GTTCTCGACC ATGCTCCTGC ACCTCCCGAC
197  LeuValArgT hrAspAsnPr oLysSerTrp TyrGluAspV alGluGlyCy
```

FIG._4A

```
651   CGGCATCCAG TGCCAGAACC CGCTCTTCAC AGAGGCTGAG CACCAGGACA
      GCCGTAGGTC ACGGTCTTGG GCGAGAAGTG TCTCCGACTC GTGGTCCTGT
214   sGlyIleGln CysGlnAsnP roLeuPheTh rGluAlaGlu HisGlnAspMet

701   TGCACAGCTA CATCGCGGCC TTCGGGGCCG TCACGGGCCT CTGCACGCTC
      ACGTGTCGAT GTAGCGCCGG AAGCCCCGGC AGTGCCCGGA GACGTGCGAG
231     HisSerTy rIleAlaAla PheGlyAlaV alThrGlyLe uCysThrLeu

751   TTCACCCTGG CCACATTCGT GGCTGACTGG CGGAACTCGA ATCGCTACCC
      AAGTGGGACC GGTGTAAGCA CCGACTGACC GCCTTGAGCT TAGCGATGGG
247   PheThrLeuA laThrPheVa lAlaAspTrp ArgAsnSerA snArgTyrPro

801   TGCTGTTATT CTCTTCTACG TCAATGCGTG CTTCTTTGTG GGCAGCATTG
      ACGACAATAA GAGAAGATGC AGTTACGCAC GAAGAAACAC CCGTCGTAAC
264    AlaValIle LeuPheTyrV alAsnAlaCy sPhePheVal GlySerIleG
                                  start clone 14

851   GCTGGCTGGC CCAGTTCATG GATGGTGCCC GCCGAGAGAT CGTCTGCCGT
      CGACCGACCG GGTCAAGTAC CTACCACGGG CGGCTCTCTA GCAGACGGCA
281   lyTrpLeuAl aGlnPheMet AspGlyAlaA rgArgGluIl eValCysArg

901   GCAGATGGCA CCATGAGGCT TGGGGAGCCC ACCTCCAATG AGACTCTGTC
      CGTCTACCGT GGTACTCCGA ACCCCTCGGG TGGAGGTTAC TCTGAGACAG
297   AlaAspGlyT hrMetArgLe uGlyGluPro ThrSerAsnG luThrLeuSe

951   CTGCGTCATC ATCTTTGTCA TCGTGTACTA CGCCCTGATG GCTGGTGTGG
      GACGCAGTAG TAGAAACAGT AGCACATGAT GCGGGACTAC CGACCACACC
314   rCysValIle IlePheValI leValTyrTy rAlaLeuMet AlaGlyValVal

1001  TTTGGTTTGT GGTCCTCACC TATGCCTGGC ACACTTCCTT CAAAGCCCTG
      AAACCAAACA CCAGGAGTGG ATACGGACCG TGTGAAGGAA GTTTCGGGAC
331     TrpPheVa lValLeuThr TyrAlaTrpH isThrSerPh eLysAlaLeu

1051  GGCACCACCT ACCAGCCTCT CTCGGGCAAG ACCTCCTACT TCCACCTGCT
      CCGTGGTGGA TGGTCGGAGA GAGCCCGTTC TGGAGGATGA AGGTGGACGA
347   GlyThrThrT yrGlnProLe uSerGlyLys ThrSerTyrP heHisLeuLeu

1101  CACCTGGTCA CTCCCCTTTG TCCTCACTGT GGCAATCCTT GCTGTGGCGC
      GTGGACCAGT GAGGGGAAAC AGGAGTGACA CCGTTAGGAA CGACACCGCG
364    ThrTrpSer LeuProPheV alLeuThrVa lAlaIleLeu AlaValAlaG

1151  AGGTGGATGG GGACTCTGTG AGTGGCATTT GTTTTGTGGG CTACAAGAAC
      TCCACCTACC CCTGAGACAC TCACCGTAAA CAAAACACCC GATGTTCTTG
381   lnValAspGl yAspSerVal SerGlyIleC ysPheValGl yTyrLysAsn

1201  TACCGATACC GTGCGGGCTT CGTGCTGGCC CCAATCGGCC TGGTGCTCAT
      ATGGCTATGG CACGCCCGAA GCACGACCGG GGTTAGCCGG ACCACGAGTA
397   TyrArgTyrA rgAlaGlyPh eValLeuAla ProIleGlyL euValLeuIl

1251  CGTGGGAGGC TACTTCCTCA TCCGAGGAGT CATGACTCTG TTCTCCATCA
      GCACCCTCCG ATGAAGGAGT AGGCTCCTCA GTACTGAGAC AAGAGGTAGT
414   eValGlyGly TyrPh LeuI leArgGlyVa lMetThrLeu PheSerIleLys
```

FIG._4B

```
1301  AGAGCAACCA CCCCGGGCTG CTGAGTGAGA AGGCTGCCAG CAAGATCAAC
      TCTCGTTGGT GGGGCCCGAC GACTCACTCT TCCGACGGTC GTTCTAGTTG
 431    SerAsnHi sProGlyLeu LeuSerGluL ysAlaAlaSe rLysIleAsn

1351  GAGACCATGC TGCGCCTGGG CATTTTTGGC TTCCTGGCCT TTGGCTTTGT
      CTCTGGTACG ACGCGGACCC GTAAAAACCG AAGGACCGGA AACCGAAACA
 447  GluThrMetL euArgLeuGl yIlePheGly PheLeuAlaP heGlyPheVal

1401  GCTCATTACC TTCAGCTGCC ACTTCTACGA CTTCTTCAAC CAGGCTGAGT
      CGAGTAATGG AAGTCGACGG TGAAGATGCT GAAGAAGTTG GTCCGACTCA
 464   LeuIleThr PheSerCysH isPheTyrAs pPhePheAsn GlnAlaGluT

1451  GGGAGCGCAG CTTCCGGGAC TATGTGCTAT GTCAGGCCAA TGTGACCATC
      CCCTCGCGTC GAAGGCCCTG ATACACGATA CAGTCCGGTT ACACTGGTAG
 481  rpGluArgSe rPheArgAsp TyrValLeuC ysGlnAlaAs nValThrIle

1501  GGGCTGCCCA CCAAGCAGCC CATCCCTGAC TGTGAGATCA AGAATCGCCC
      CCCGACGGGT GGTTCGTCGG GTAGGGACTG ACACTCTAGT TCTTAGCGGG
 497  GlyLeuProT hrLysGlnPr oIleProAsp CysGluIleL ysAsnArgPr

1551  GAGCCTTCTG GTGGAGAAGA TCAACCTGTT TGCCATGTTT GGAACTGGCA
      CTCGGAAGAC CACCTCTTCT AGTTGGACAA ACGGTACAAA CCTTGACCGT
 514  oSerLeuLeu ValGluLysI leAsnLeuPh eAlaMetPhe GlyThrGlyIle

1601  TCGCCATGAG CACCTGGGTC TGGACCAAGG CCACGCTGCT CATCTGGAGG
      AGCGGTACTC GTGGACCCAG ACCTGGTTCC GGTGCGACGA GTAGACCTCC
 531    AlaMetSe rThrTrpVal TrpThrLysA laThrLeuLe uIleTrpArg

1651  CGTACCTGGT GCAGGTTGAC TGGGCAGAGT GACGATGAGC CAAAGCGGAT
      GCATGGACCA CGTCCAACTG ACCCGTCTCA CTGCTACTCG GTTTCGCCTA
 547  ArgThrTrpC ysArgLeuTh rGlyGlnSer AspAspGluP roLysArgIle

1701  CAAGAAGAGC AAGATGATTG CCAAGGCCTT CTCTAAGCGG CACGAGCTCC
      GTTCTTCTCG TTCTACTAAC GGTTCCGGAA GAGATTCGCC GTGCTCGAGG
 564   LysLysSer LysMetIleA laLysAlaPh eSerLysArg HisGluLeuL

1751  TGCAGAACCC AGGCCAGGAG CTGTCCTTCA GCATGCACAC TGTGTCCCAC
      ACGTCTTGGG TCCGGTCCTC GACAGGAAGT CGTACGTGTG ACACAGGGTG
 581  euGlnAsnPr oGlyGlnGlu LeuSerPheS erMetHisTh rValSerHis

1801  GACGGGCCCG TGGCGGGCTT GGCCTTTGAC CTCAATGAGC CCTCAGCTGA
      CTGCCCGGGC ACCGCCCGAA CCGGAAACTG GAGTTACTCG GGAGTCGACT
 597  AspGlyProV alAlaGlyLe uAlaPheAsp LeuAsnGluP roSerAlaAs

1851  TGTCTCCTCT GCCTGGGCCC AGCATGTCAC CAAGATGGTG GCTCGGAGAG
      ACAGAGGAGA CGGACCCGGG TCGTACAGTG GTTCTACCAC CGAGCCTCTC
 614  pValSerSer AlaTrpAlaG lnHisValTh rLysMetVal AlaArgArgGly
                          end clone 5

1901  GAGCCATACT GCCCCAGGAT ATTTCTGTCA CCCCTGTGGC AACTCCAGTG
      CTCGGTATGA CGGGGTCCTA TAAAGACAGT GGGGACACCG TTGAGGTCAC
 631    AlaIleLe uProGlnAsp IleSerValT hrProValAl aThrProVal
```

FIG._4C

```
1951  CCCCCAGAGG AACAAGCCAA CCTGTGGCTG GTTGAGGCAG AGATCTCCCC
      GGGGGTCTCC TTGTTCGGTT GGACACCGAC CAACTCCGTC TCTAGAGGGG
 647  ProProGluG luGlnAlaAs nLeuTrpLeu ValGluAlaG luIleSerPro

2001  AGAGCTGCAG AAGCGCCTGG GCCGGAAGAA GAAGAGGAGG AAGAGGAAGA
      TCTCGACGTC TTCGCGGACC CGGCCTTCTT CTTCTCCTCC TTCTCCTTCT
 664    GluLeuGln LysArgLeuG lyArgLysLy sLysArgArg LysArgLysL

2051  AGGAGGTGTG CCCGCTGGCG CCGCCCCCTG AGCTTCACCC CCCTGCCCCT
      TCCTCCACAC GGGCGACCGC GGCGGGGGAC TCGAAGTGGG GGGACGGGGA
 681  ysGluValCy sProLeuAla ProProProG luLeuHisPr oProAlaPro

2101  GCCCCCAGTA CCATTCCTCG ACTGCCTCAG CTGCCCCGGC AGAAATGCCT
      CGGGGGTCAT GGTAAGGAGC TGACGGAGTC GACGGGGCCG TCTTTACGGA
 697  AlaProSerT hrIleProAr gLeuProGln LeuProArgG lnLysCysLe

2151  GGTGGCTGCA GGTGCCTGGG GAGCTGGGGA CTCTTGCCGA CAGGGAGCGT
      CCACCGACGT CCACGGACCC CTCGACCCCT GAGAACGGCT GTCCCTCGCA
 714  uValAlaAla GlyAlaTrpG lyAlaGlyAs pSerCysArg GlnGlyAlaTrp

2201  GGACCCTGGT CTCCAACCCA TTCTGCCCAG AGCCCAGTCC CCCTCAGGAT
      CCTGGGACCA GAGGTTGGGT AAGACGGGTC TCGGGTCAGG GGGAGTCCTA
 731    ThrLeuVa lSerAsnPro PheCysProG luProSerPr oProGlnAsp

2251  CCATTTCTGC CCAGTGCACC GGCCCCCGTG GCATGGGCTC ATGGCCGCCG
      GGTAAAGACG GGTCACGTGG CCGGGGGCAC CGTACCCGAG TACCGGCGGC
 747  ProPheLeuP roSerAlaPr oAlaProVal AlaTrpAlaH isGlyArgArg

2301  ACAGGGCCTG GGGCCTATTC ACTCCCGCAC CAACCTGATG GACACAGAAC
      TGTCCCGGAC CCCGGATAAG TGAGGGCGTG GTTGGACTAC CTGTGTCTTG
 764   GlnGlyLeu GlyProIleH isSerArgTh rAsnLeuMet AspThrGluL

2351  TCATGGATGC AGACTCGGAC TTCTGAGCCT GCAGAGCAGG ACCTGGGACA
      AGTACCTACG TCTGAGCCTG AAGACTCGGA CGTCTCGTCC TGGACCCTGT
 781  euMetAspAl aAspSerAsp Phe
                              Stop

2401  GGAAAGAGAG GAACCAATAC CTTCAAGGCT CTTCTTCCTC ACCGAGCATG
      CCTTTCTCTC CTTGGTTATG GAAGTTCCGA GAAGAAGGAG TGGCTCGTAC

2451  CTTCCCTAGG ATCCCGTCTT CCAGAGAACC TGTGGGCTGA CTGCCCTCCG
      GAAGGGATCC TAGGGCAGAA GGTCTCTTGG ACACCCGACT GACGGGAGGC

2501  AAGAGAGTTC TGGATGTCTG GCTCAAAGCA GCAGGACTGT GGGAAAGAGC
      TTCTCTCAAG ACCTACAGAC CGAGTTTCGT CGTCCTGACA CCCTTTCTCG

2551  CTAACATCTC CATGGGGAGG CCTCACCCCA GGGACAGGGC CCTGGAGCTC
      GATTGTAGAG GTACCCCTCC GGAGTGGGGT CCCTGTCCCG GGACCTCGAG

2601  AGGGTCCTTG TTTCTGCCCT GCCAGCTGCA GCCTGGTTGG CAGCATCTGC
      TCCCAGGAAC AAAGACGGGA CGGTCGACGT CGGACCAACC GTCGTAGACG
```

FIG._4D

```
2651  TCCATCGGGG CAGGGGGTAT GCAGAGCTTG TGGTGGGGCA GGAACGGTGG
      AGGTAGCCCC GTCCCCCATA CGTCTCGAAC ACCACCCCGT CCTTGCCACC

2701  AGGCAGAGGT GACAGTTCCC AGAGTGGGCT TTGGTGGCCA GGGAGGCAGC
      TCCGTCTCCA CTGTCAAGGG TCTCACCCGA AACCACCGGT CCCTCCGTCG

2751  CTAGCCTATG TCTGGCAGAT GAGGGCTGGC TGCCGTTTTC TGGGCTGATG
      GATCGGATAC AGACCGTCTA CTCCCGACCG ACGGCAAAAG ACCCGACTAC

2801  GGTGCCCTTT CCTGGCAGTC TCAGTCCAAA AGTGTTGACT GTGTCATTAG
      CCACGGGAAA GGACCGTCAG AGTCAGGTTT TCACAACTGA CACAGTAATC

2851  TCCTTTGTCT AAGTAGGGCC AGGGCACCGT ATTCCTCTCC CAGGTGTTTG
      AGGAAACAGA TTCATCCCGG TCCCGTGGCA TAAGGAGAGG GTCCACAAAC

2901  TGGGGCTGGA AGGACCTGCT CCCACAGGGG CCATGTCCTC TCTTAATAGG
      ACCCCGACCT TCCTGGACGA GGGTGTCCCC GGTACAGGAG AGAATTATCC

2951  TGGCACTACC CCAAACCCAC CG
      ACCGTGATGG GGTTTGGGTG GC
```

FIG._4E

```
hSmo       1  - - - - - - - - M A A A R P A R G P E - - - - - - - - - - - - - L P - - - L L G L L L L L L G - -
rat.smo    1  - - - - - - - - M A A G R P V R G P E - - - - - - - - - - - - - L A P R R L L Q L L L V L L G - -
dros.smo   1  M H X R T A E Q E T G I Q P I K I H T R Q L F N D F Y K R M Q Y L N F P R M P N I M M F L E V A I L

hSmo      25  - - - D P G R G A A S S G N A T G P G P R S A G G S A R R S A A V T G P - - - - - - - - - - - - - P P
rat.smo   28  - - - G R G R G A A L S G N V T G P G P R S A G G S A R R N A P V T S P - - - - - - - - - - - - - P P
dros.smo  51  C L W V V A D A S A S S A K F G S T T P A S A Q Q S D V E L E P I N G T L N Y R L Y A K K G R D D K

hSmo      60  P - L S - - - - - - - H C G R A A P C E P L R Y - - N V C L G S V L P Y G A T S T L L A G D S D S Q
rat.smo   63  P L L S - - - - - - - H C G R A A H C E P L R Y - - N V C L G S A L P Y G A T T T L L A G D S D S Q
dros.smo 101  P W F D G L D S R H I Q C V R R A R C Y P T S N A T N T C F G S K L P Y E L S S L D L T - D F H T E

hSmo     100  E E A H G K L V L W S G L R N A P R C W A V I Q P L L C A V Y M P K C E - - - - N D R V E L P S R T
rat.smo  104  E E A H S K L V L W S G L R N A P R C W A V I Q P L L C A V Y M P K C E - - - - N D R V E L P S R T
dros.smo 150  K E L N D K L N D Y Y A L K H V P K C W A A I Q P F L C A V F K P K C E K I N G E D M V Y L P S Y E

hSmo     146  L C Q A T R G P C A I V E R E R G W P D F L R C T P D R F P E G C T N E V Q N I K F N S S G Q C E V
rat.smo  150  L C Q A T R G P C A I V E R E R G W P D F L R C T P D H F P E G C P N E V Q N I K F N S S G Q C E A
dros.smo 200  M C R I T M E P C R I L Y N T T F F P K F L R C N E T L F P T K C T N G A R G M K F N G T G Q C L S
```

FIG._5A

```
hSmo       196  PLVRTDNPKSWYEDVEGCGIQCQNPLFTEAEHQDMHSYIAAFGAVTGLCT
rat.smo    200  PLVRTDNPKSWYEDVEGCGIQCQNPLFTEAEHQDMHSYIAAFGAVTGLCT
dros.smo   250  PLVPTDTSASYYPGIEGCGVRCKDPLYTDDEHRQIHKLIGWAGSICLLSN

hSmo       246  LFTLATFVADWRNSNRYPAVILFYVNACFFVGSIGWLAQFMDGARREIVC
rat.smo    250  LFTLATFVADWRNSNRYPAVILFYVNACFFVGSIGWLAQFMDGARREIVC
dros.smo   300  LFVVSTFFIDWKNANKYPAVIVFYINLCFLIACVGWLLQFTSGSREDIVC

hSmo       296  RADGTMRLGEPTSNETLSCVIIFVIVYYALMAGVVWFVVLTYAWHTSFKA
rat.smo    300  RADGTMRFGEPTSSETLSCVIIFVIVYYALMAGVVWFVVLTYAWHTSFKA
dros.smo   350  RKDGTLRHSEPTAGENLSCIVIFVLVYYFLTAGMVWFVFLTYAWH--WRA

hSmo       346  LGTTYQPLSGKTSYFHLLTWSLPFVLTVAILAVAQVDGDSVSGICFVGYK
rat.smo    350  LGTTYQPLSGKTSYFHLLTWSLPFVLTVAILAVAQVDGDSVSGICFVGYK
dros.smo   398  MGHVQDRIDKKGSYFHLVAWSLPLVLTITTMAFSEVDGNSIVGICFVGYI

hSmo       396  NYRYRAGFVLAPIGLVLIVGGYFLIRGVMTLFSIKSNHPGLLSEKAASKI
rat.smo    400  NYRYRAGFVLAPIGLVLIVGGYFLIRGVMTLFSIKSNHPGLLSEKAASKI
dros.smo   448  NHSMRAGLLLGPLCGVILIGGYFITRGMVMLFGLKHFANDIKSTSASNKI
```

FIG._5B

```
hSmo      446  NETMLRLGIFGFLAFGFVLITFSCHFYDFFNQAEWERSFRDYVLCQANVT
rat.smo   450  NETMLRLGIFGFLAFGFVLITFSCHFYDFFNQAEWERSFRDYVLCQANVT
dros.smo  498  HLIIMRMGVCALLTLVFILVAIACHVTEFRHADEWAQSFRQFIIC--KIS

hSmo      496  IGLPTKQPIPDCEIKNRPSLLVEKINLFAMFGTGIAMSTWVWTKATLLIW
rat.smo   500  IGLPTKKPIPDCEIKNRPSLLVEKINLFAMFGTGIAMSTWVWTKATLLIW
dros.smo  546  SVFEEK---SSCRIENRPSVGVLQLHLLCLFSSGIVMSTWCWTPSSIETW

hSmo      546  RRTWCRLTGQSDDEPKRIKKSKMIAKAFSKRHELLQNPGQELSFSMHTVS
rat.smo   550  RRTWCRLTGHSDDEPKRIKKSKMIAKAFSKRRELLQNPGQELSFSMHTVS
dros.smo  593  KRYIRKKCGKEVVEEVKMPKHKVIAQTWAKRKD-FEDKGR-LSITLYN-T

hSmo      596  HDGPVAGLAFDLNE----PSADVSSAWAQHVTKMVARR----GAILPQDI
rat.smo   600  HDGPVAGLAFELNE----PSADVSSAWAQHVTKMVARR----GAILPQDV
dros.smo  640  HTDPV-GLNFDVNDLNSSETNDISSTWAAYLPQCVKRRMALTGAATGNSS

hSmo      638  SVTPVATPVPPEEQANLWL---------VEAEIS---PELQKRLG-----
rat.smo   642  SVTPVATPVPPEEQANLWL---------VEAEIS---PELEKRLG-----
dros.smo  689  SHGPRKNSLDSEISVSVRHVSVESRRNSVDSQVSVKIAEMKTKVASRSRG
```

FIG._5C

```
hSmo      671  . . . . . . . . R K K K R R K . . . . . . . . . . . R K K E . . . . . . . . . . . . . . . . . . . .
rat.smo   675  . . . . . . . . R K K K R R K . . . . . . . . . . . R K K E . . . . . . . . . . . . . . . . . . . .
dros.smo  739  K H G G S S S N R R T Q R R R D Y I A A A T G K S S R R R E S S T S V E S Q V I A L K K T T Y P N A

hSmo      682  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . V C P L
rat.smo   686  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . V C P L
dros.smo  789  S H K V G V F A H H S S K K Q H N Y T S S M K R R T A N A G L D P S I L N E F L Q K N G D F I F P F

hSmo      686  A P P P E L H P P A P A P S T - - I P R L P Q L . . . . . . . . . . . . . . . . . . . . . . . . P R
rat.smo   690  G P A P E L H H S A P V P A T S A V P R L P Q L . . . . . . . . . . . . . . . . . . . . . . . . P R
dros.smo  839  L Q N Q D M S S S S E E D N S R A S Q K I Q D L N V V V K Q Q E I S E D D H D G I K I E E L P N S K

hSmo      710  Q . . . . . . . K C L V A A G A W G A G D S C R Q G A W . . . . . . . . . . . . . . . . . . . . . .
rat.smo   716  Q . . . . . . . K C L V A A N A W G T G E P C R Q G A W . . . . . . . . . . . . . . . . . . . . . .
dros.smo  889  Q V A L E N F L K N I K K S N E S N S N R H S R N S A R S Q S K K S Q K R H L K N P A A D L D F R K
```

FIG._5D

```
hSmo      731  - - - - - - - - - - - - - - - - - - - - - - - - - T L V S N P F C P - - - - - - - - - - - - - - - E
rat.smo   737  - - - - - - - - - - - - - - - - - - - - - - - - - T V V S N P F C P - - - - - - - - - - - - - - - E
dros.smo  939  D C V K Y R S N D S L S C S S E E L D V A L D V G S L L N S S F S G I S M G K P H S R N S K T S C D

hSmo      741  P S P P Q D P F - - L P S - - - - - - - - - - - - - - - A P A P V A W A H G R R Q G L G P I - - - - - -
rat.smo   747  P S P H Q D P F - - L P G - - - - - - - - - - - - - - - A S A P R V W A Q G R L Q G L G S I - - - - - -
dros.smo  989  V G I Q A N P F E L V P S Y G E D E L Q Q A M R L L N A A S R Q R T E A A N E D F G G T E L Q G L L

hSmo      770  - H S R T N L M D T E L M D A D S D F - - - - - - - -
rat.smo   776  - H S R T N L M E A E L L D A D S D F - - - - - - - -
dros.smo  1039 G H S H R H Q R E P T F M S E S D K L K M L L L P S K
```

FIG._5E

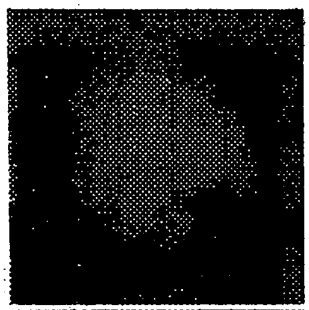


FIG._6A

FIG._6B

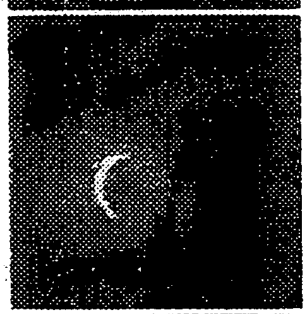


FIG._6C

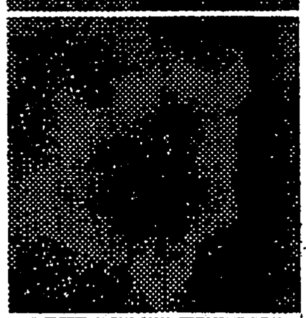


FIG._6D

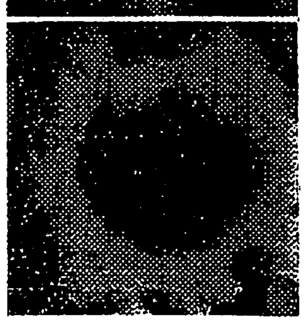


FIG._6E

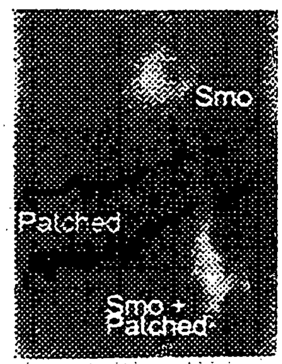


FIG._7A

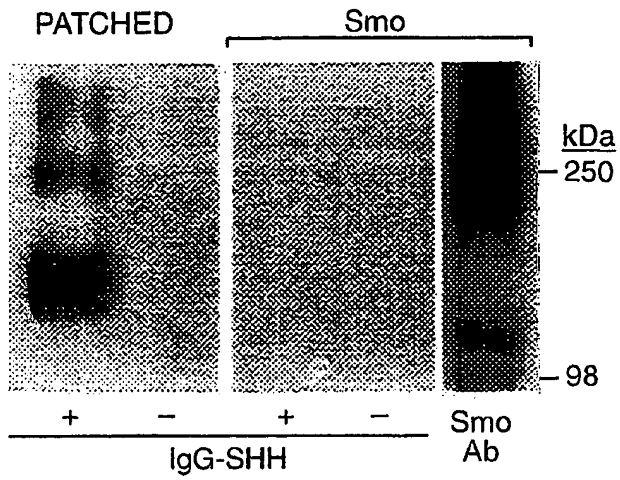


FIG._6F

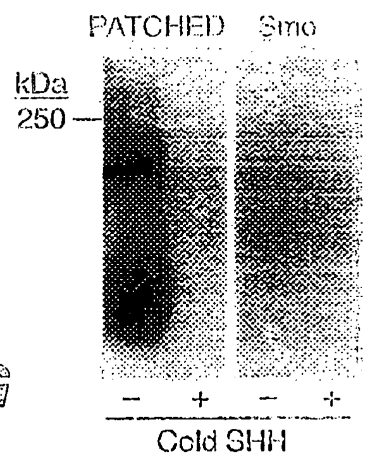


FIG._6G

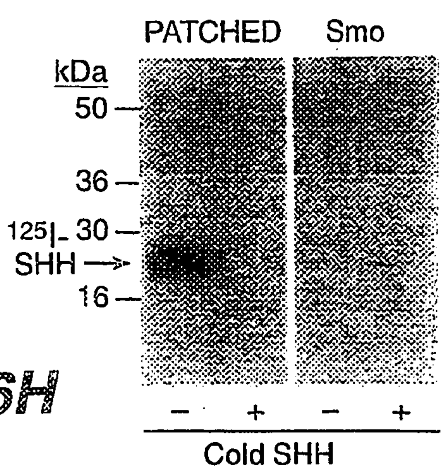


FIG._6H

FIG._6I

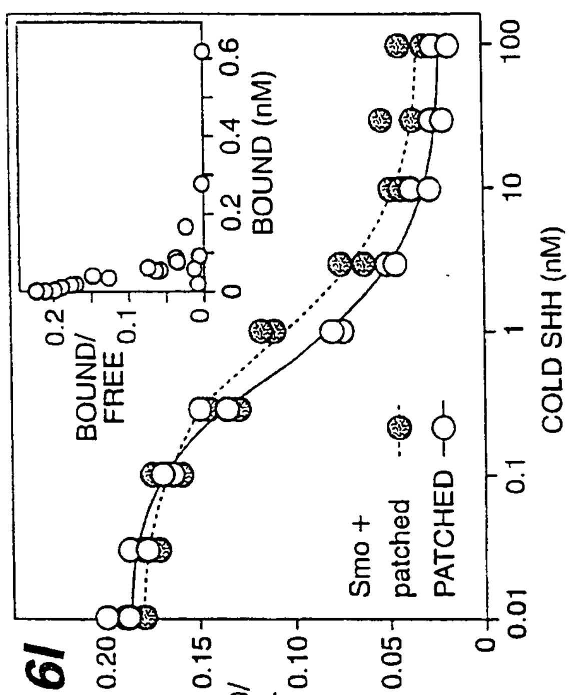
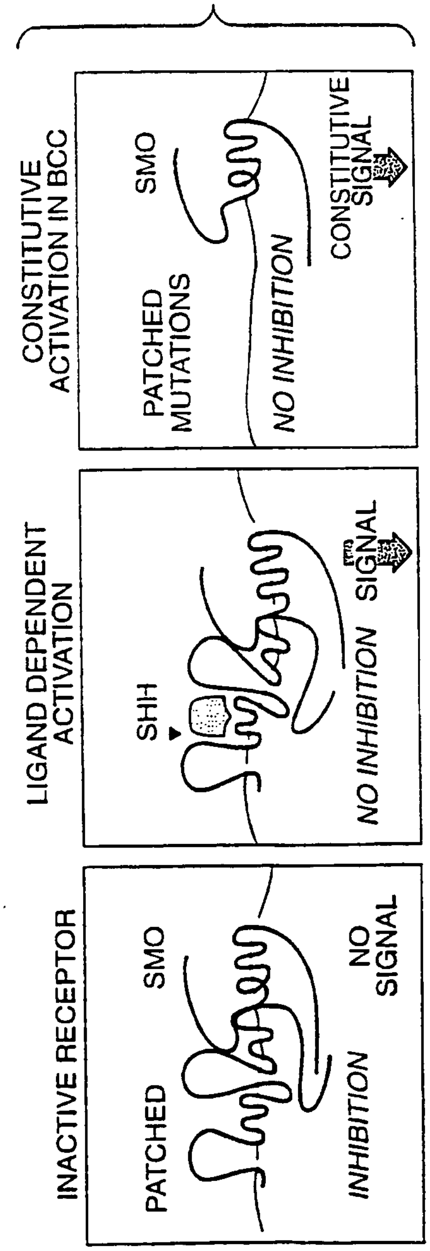

BOUND/
TOTAL
0.20
0.15
0.10
0.05
0
0.01
0.1
1
10
100
COLD SHH (nM)
Smo +
patched
PATCHED
BOUND/
FREE
0.2
0.1
0
0
0.2
0.4
0.6
BOUND (nM)

FIG._9


INACTIVE RECEPTOR
PATCHED
SMO
INHIBITION
NO
SIGNAL
LIGAND DEPENDENT
ACTIVATION
SHH
NO INHIBITION
SIGNAL
CONSTITUTIVE
ACTIVATION IN BCC
PATCHED
MUTATIONS
SMO
NO INHIBITION
CONSTITUTIVE
SIGNAL

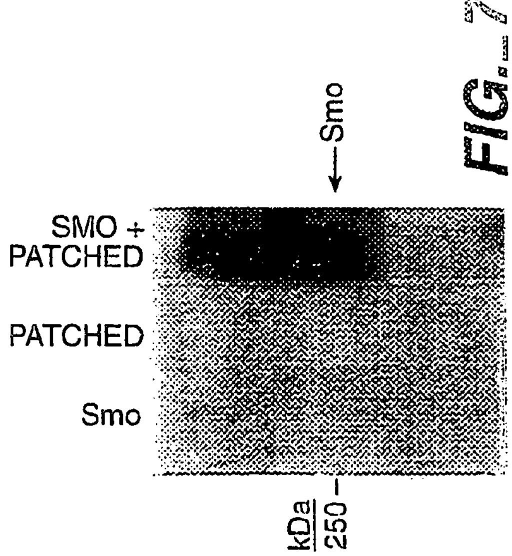


FIG._7B

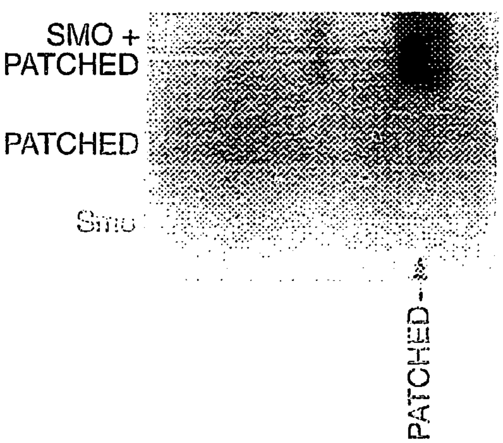


FIG._7C

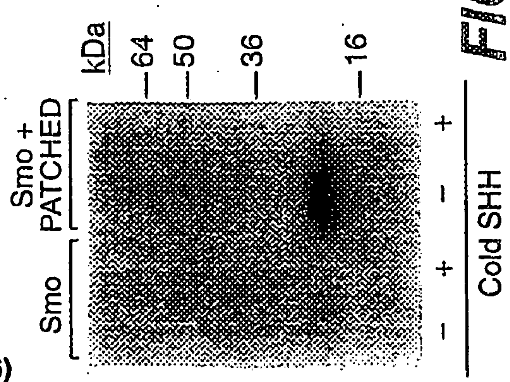


FIG._7D

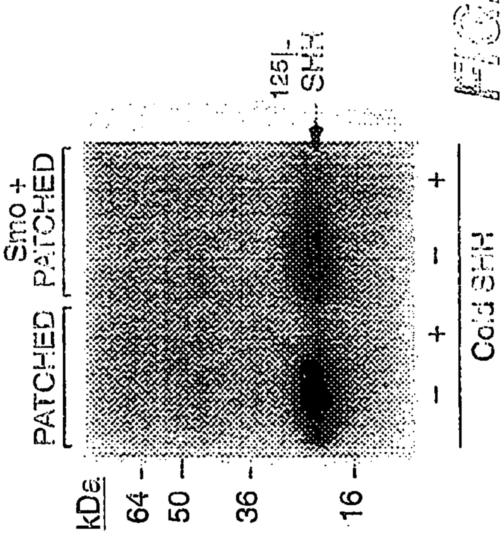


FIG._7E

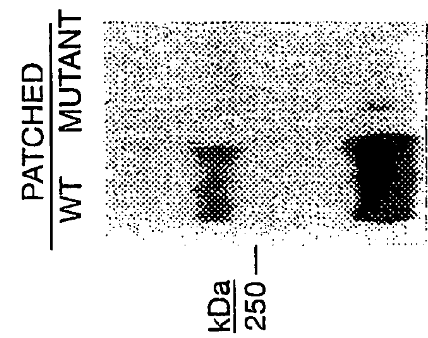


FIG._8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/17433

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C12N15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VAN DEN HEUVEL ET AL.: "Smoothened encodes a receptor- like serpentine protein required for hedgehog signalling" NATURE, vol. 382, 8 August 1996, pages 547-51, XP002054237 cited in the application ---	
P,X	D.M.STONE ET AL.: "The tumor suppressor gene patched encodes a candidate receptor for sonic hedgehog" NATURE, vol. 384, 14 November 1996, pages 129-134, XP002054238 see figure 1 -----	1-32

☐ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
2 February 1998	20. 02. 98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Deffner, C-A